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Function of the regulator OmpR in the modulation of the outer membrane proteome of *Yersinia enterocolitica*

Rozprawa doktorska
w zakresie nauk biologicznych
dyscyplinie biologii

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Funkcja regulatora OmpR w modulowaniu proteomu błony zewnętrznej *Yersinia enterocolitica*

Spis treści

Lista prac wchodzących w skład rozprawy doktorskiej

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Streszczenie

Yersinia enterocolitica to gramujemna bakteria wywołująca jersinozę – ostrą lub przewlekłą, odzwierzęcą chorobę zakaźną, która może przyjmować różne postaci kliniczne, najczęściej żołądkowo-jelitowe. Pałeczki *Y. enterocolitica* są zdolne do kolonizacji wielu różnorodnych nisz w obrębie organizmu gospodarza, a także w środowisku naturalnym (w wodzie i glebie). Kluczową rolę w procesie patogenezy oraz adaptacji do zmiennych warunków środowiska pełnią białka błony zewnętrznej (OM), a wśród nich czynniki wirulencji, m.in. adhezyny YadA, Ail, Inv, Myf, białka systemu sekrecji III typu Ysc-Yop, a także poryny dyfuzji ogólnej i specyficznej, receptory związane z transportem substancji odżywczych oraz jonów czy systemy wyrzutu *efflux*.

Synteza czynników wirulencji, jak i odpowiedź adaptacyjna bakterii patogennych na zmienne warunki środowiska, tj. temperaturę, pH, osmolarność, dostępność składników odżywczych/jonów, a także na obecność substancji toksycznych, pozwalają przeżyć w określonej niszy ekologicznej oraz skutecznie konkurować z naturalnym mikrobiomem.

Dwuskładnikowe systemy transdukcji sygnału (TCS) to szlaki regulacyjne, które umożliwiają bakteriom odbieranie oraz reagowanie na liczne zewnętrzne sygnały poprzez modulację ekspresji odpowiednich genów. Archetypem TCS jest szlak sygnałowy EnvZ/OmpR niepatogennej *Escherichia coli* K-12 uczestniczący w osmoregulacji ekspresji genów białek porynowych OmpC i OmpF. System EnvZ/OmpR składa się z transbłonowej kinazy histydynowej EnvZ, która odbierając sygnał ze środowiska, przenosi go w postaci grupy fosforanowej na partnerskie białko cytoplazmatyczne – regulator odpowiedzi OmpR. Ufosforylowane białko OmpR wiąże się z regionem promotorowym genu *ompC* oraz *ompF* i w sposób pozytywny lub negatywny reguluje ich transkrypcję.

Funkcja białka OmpR w regulacji ekspresji genów jest od lat przedmiotem intensywnych badań u różnych gatunków bakterii, w tym u *Y. enterocolitica*. Wyniki sugerują, że regulator OmpR może pełnić różnorodne, często odmienne funkcje, także specyficzne dla określonego gatunku bakterii. Klasycznym podejściem stosowanym do identyfikacji celów działania OmpR było dotychczas badanie fizjologicznych konsekwencji delecji genu regulatora. Wprowadzenie technik transkryptomicznych oraz proteomicznych umożliwiło kompleksowe spojrzenie na funkcję OmpR u bakterii.

Badania prezentowane w pracy doktorskiej koncentrowały się na poznaniu funkcji regulatora OmpR *Y. enterocolitica* w modulowaniu składników proteomu błony zewnętrznej

oraz wyjaśnieniu roli OmpR w regulacji ekspresji wytypowanych genów na poziomie transkrypcyjnym.

Do identyfikacji białek błony zewnętrznej, regulowanych przez białko OmpR zastosowano różnicową analizę proteomiczną metodą *shotgun*. W pierwszym etapie prac dokonano optymalizacji protokołu izolacji białek OM, wykorzystując szereg detergentów w zmiennych kombinacjach i stężeniach. Optymalizowano także warunki trawienia trypsyną uzyskanych frakcji białkowych. Wyniki analiz LC-MS/MS przeprowadzanych podczas testowania protokołów izolacji białek OM, pozwoliły na wybór optymalnej metodyki, którą zastosowano we właściwych eksperymentach.

Frakcje białkowe błon, wzbogacone o białka OM, uzyskane ze szczepów różniących się obecnością białka OmpR, tj. dzikiego i mutanta Δ*ompR,* hodowanych w różnych warunkach temperatury, osmolarności oraz pH, analizowano w systemie HPLC sprzężonym z tandemowym spektrometrem mas Orbitrap Velos. Wyniki LC-MS/MS, po bioinformatycznej, jakościowej i ilościowej analizie danych, pozwoliły na utworzenie list białek różnicowych.

Na podstawie danych MS wykazano, że OmpR, w określonych warunkach środowiska, reguluje pozytywnie lub negatywnie poziom 120 białek, w tym związanych pośrednio lub bezpośrednio z błoną zewnętrzną, które pełnią w komórce różnorodne funkcje. Wśród nich zidentyfikowano znane, opisane wcześniej, jak i nowe OmpR-zależne białka, w tym także charakterystyczne dla bakterii z rodzaju *Yersinia*. Białka różnicowe przyporządkowano do określonych kategorii procesów biologicznych, w których uczestniczą. Wykazano wpływ OmpR na szereg białek biorących udział w dyfuzji, transporcie aktywnym, czynnym usuwaniu substancji toksycznych, w utrzymaniu homeostazy żelazowej, a także organizacji błony zewnętrznej oraz patogenezie. Ocena zmian w składzie proteomu pozwoliła na określenie biologicznej funkcji OmpR u *Y. enterocolitica* i jego kluczowej roli w adaptacji do zmiennych warunków środowiska.

Zdefiniowany OmpR-zależny proteom stanowił punkt wyjścia do analiz *in silico* w celu identyfikacji sekwencji DNA wiążących OmpR. Potencjalne motywy wiązania białka OmpR wyznaczono w obrębie sekwencji regulatorowych genów *cycA, dcuA, fecA, fepA, hemR, kdgM2, myfC, ompW, scrY* i *yadA*, a także *acrA, fadL, ompC, ompF, ompX*, *tppB*, które jako geny regulonu OmpR zidentyfikowano wcześniej u *E. coli* i *Salmonella*.

Do szczegółowych badań genetycznych i biochemicznych mających na celu poznanie mechanizmu OmpR-zależnej regulacji, wytypowano *yadA*, *hemR* oraz *kdgM2*, kodujące odpowiednio, czynnik wirulencji YadA warunkujący adhezję i oporność komórki na bakteriobójcze działanie surowicy, receptor HemR niezbędny dla przyswajania hemu,

a także białko KdgM2, ortolog poryn specyficznych dla oligogalakturonianów (OGA) – produktów degradacji pektyn, u patogenów roślin, w tym *Dickeya dadantii*. Dla białka KdgM2 zanotowano ponad 100-krotny, tj. najwyższy parametr krotności zmiany, wśród OmpR-zależnych składników proteomu OM.

Badania nad rolą OmpR w regulacji ekspresji genów YadA i HemR rozpoczęto od analiz Western blot, które wykazały wyższy poziom obu białek w szczepie niesyntezującym regulatora OmpR i tym samym potwierdziły wyniki proteomiczne. Analizy aktywności promotorów wskazały, że białko OmpR negatywnie reguluje poziom ekspresji genów *yadA* i *hemR*. Badania opóźnienia tempa migracji kompleksów nukleoproteinowych w natywnym żelu poliakryloamidowym tzw. testy EMSA udowodniły, że OmpR-zależna regulacja transkrypcji *yadA* wynika z bezpośredniego oddziaływania regulatora ze specyficzną sekwencją wiążącą. W przypadku *hemR* zaproponowano mechanizm, w którym OmpR negatywnie, ale pośrednio reguluje ekspresję *hemR*.

Punktem wyjścia do prac nad określeniem funkcji białka OmpR w regulacji ekspresji *kdgM2* były analizy bioinformatyczne sekwencji genomów bakterii. Pozwoliły one na zidentyfikowanie u *Y. enterocolitica* klastrów genów, charakterystycznych dla fitopatogenów, które są związane z transportem oraz depolimeryzacją produktów degradacji pektyn, w tym kodujących poryny KdgM2 i KdgM1, specyficzne dla OGA oraz regulator KdgR. Ponadto, analizy *in silico* wykazały brak genów zewnątrzkomórkowych enzymów pektynolitycznych, typowych dla patogenów roślin, co potwierdzono w badaniach *in vitro* i *in vivo*.

W kolejnym etapie pracy przeprowadzono szereg badań z wykorzystaniem skonstruowanych mutantów delecyjnych, fuzji reporterowych, RT-qPCR, SDS-PAGE oraz EMSA, które dowiodły, że OmpR wykazuje bezpośredni, ale odwrotny efekt regulatorowy na poziom białek KdgM2 i KdgM1, w wyniku negatywnej regulacji transkrypcji *kdgM2* oraz pozytywnej *kdgM1*. W toku badań ustalono także, że KdgR pełni funkcję represora genów szlaku pektynolizy, a regulator OmpR może wpływać na ekspresję *kdgM2* i *kdgM1*, w sposób pośredni, poprzez negatywną regulację transkrypcji genu *kdgR*.

Dodatkowo, badania biologicznej funkcji KdgM2 pozwoliły wnioskować o znaczeniu tego białka w przepuszczalności błony zewnętrznej *Y. enterocolitica*.

Podsumowując, przedstawiona rozprawa doktorska stanowi pierwszą, kompleksową charakterystykę roli regulatora OmpR w modulowaniu proteomu błony zewnętrznej *Y. enterocolitica*. Zidentyfikowano nieznane wcześniej geny regulonu OmpR i wykazano, że OmpR regulując ich transkrypcję (pozytywnie lub negatywnie) pełni ważną rolę

adaptacyjną. Prezentowane wyniki sugerują, że OmpR jako integrator wielu komórkowych procesów, może decydować o wyborze strategii życiowej *Y. enterocolitica*, związanej z saprofityczną lub patogenną formą życia bakterii.

Summary

Yersinia enterocolitica is a gram-negative bacterium that causes yersiniosis – an acute or chronic foodborne disease manifested by a variety of clinical symptoms, especially gastrointestinal. *Y. enterocolitica* exhibits a dual lifestyle: it can colonize many different niches within a host organism and is also found in water and soil in the natural environment. Proteins of the outer membrane (OM), including the adhesins YadA, Ail, Inv and Myf, the Ysc-Yop type III secretion system, general diffusion and specific porins, nutrient and ion receptors, and efflux pump components, play key roles in *Y. enterocolitica* pathogenesis and adaptation to changing environmental conditions.

The synthesis of virulence factors as well as the adaptive response of bacterial pathogens to variable environmental conditions (i.e. temperature, pH, osmolarity, availability of nutrients/ions), and to the presence of toxic substances, allow them to survive in a specific ecological niche and efficiently compete with the natural microbiome.

Two-component signal transduction systems (TCS) permit bacteria to recognize and respond to diverse environmental stimuli by modulating gene expression. The archetype of the TCS is EnvZ/OmpR, involved in the osmoregulation of OmpC and OmpF porin expression in non-pathogenic *Escherichia coli* K-12. The EnvZ/OmpR system consists of transmembrane histidine kinase EnvZ, which receives stimuli from the environment and transfers this signal as a phosphoryl group to the cytoplasmic response regulator OmpR. Phosphorylated OmpR then binds to the regulatory regions of the *ompC* and *ompF* genes to either activate or repress transcription.

The function of the OmpR protein in many bacterial species, including *Y. enterocolitica*, has been the subject of intense research for years. The results of these studies suggest that the regulator OmpR can perform various, often different functions, some of which are species-specific. The classical approach to identify members of the OmpR regulon was based on analysis of the physiological consequences of the loss of the OmpR protein. Recently, high-throughput methods, such as transcriptomics and proteomics, have enabled efforts to gain a more comprehensive view of the role of OmpR.

The aim of this doctoral research project was to investigate the function of *Y. enterocolitica* OmpR in the modulation of the OM proteome and to decipher its role in regulating the expression of selected genes at the transcriptional level.

Differential proteomic analysis using the shotgun strategy was applied to identify OM proteins subject to regulation by OmpR. As a first step, a protocol for the isolation

of *Y. enterocolitica* OM proteins was developed and optimized using combinations of detergents at different concentrations. The optimal conditions for trypsin digestion of the isolated proteins were also determined. LC-MS/MS analysis of samples obtained during protocol optimization assisted selection of the methodology that was used in the proper experiments.

Membrane protein fractions enriched in OM proteins, obtained from *Y. enterocolitica* strains differing in the presence of OmpR protein (i.e. the wild-type and a Δ*ompR* mutant) grown under different temperature, osmolarity and pH conditions, were analyzed by HPLC coupled with an Orbitrap Velos mass spectrometer. Bioinformatic analysis of the qualitative and quantitative proteomic data was then used to create lists of differentially-expressed proteins.

The results of the proteomic analysis indicated that under specific environmental conditions, OmpR affects (both positively and negatively), the production of 120 proteins, including integral and OM-associated proteins, which serve a variety of functions. Among them are well-characterized OmpR targets as well as newly identified OmpR-dependent proteins, including some that are *Yersinia* specific. The differentially-expressed proteins were grouped into several categories according to the biological processes in which they participate. This showed that OmpR influences the production of a number of proteins involved in diffusion, active transport, removal of toxic substances, maintenance of iron homeostasis as well as the organization of the OM and pathogenesis. Evaluation of changes in the composition of the proteome helped to elucidate the biological function of OmpR in *Y. enterocolitica* and highlight its key role in physiological adaptations necessary for growth in highly variable environments.

The defined OmpR-dependent proteome was the starting point for an *in silico* analysis to identify OmpR-binding DNA sequences. Putative OmpR-binding motifs were recognized in the promoter regions of the *Y. enterocolitica* genes *cycA, dcuA, fecA, fepA, hemR, kdgM2, myfC, ompW, scrY* and *yadA*, as well as in previously identified OmpR-regulated genes in *Salmonella* and *E. coli*, i.e. *acrA, fadL, ompC, ompF, ompX* and *tppB.*

For detailed genetic and biochemical studies on the mechanism of OmpR-mediated regulation, the genes *yadA, hemR* and *kdgM2* were chosen. The selected genes encode virulence factor YadA, which is crucial for adhesion and resistance to the bactericidal activity of serum, HemR, a receptor essential for the acquisition of heme, and KdgM2, an ortholog of porins involved in the uptake of pectin degradation products (i.e. oligogalacturonides, OGA) by plant pathogens such as *Dickeya dadantii.* The most impressive regulatory impact

of OmpR was on the abundance of porin KdgM2, which displayed a more than 100-fold change.

To confirm the role of OmpR in modulating YadA and HemR proteins levels, Western blot analysis was performed. In agreement with the proteomic results the absence of OmpR resulted in increases in YadA and HemR. Furthermore, promoter activity analysis indicated that the OmpR protein negatively regulates *yadA* and *hemR* transcription. Electrophoretic mobility shift assays (EMSA) suggested that OmpR-dependent regulation of *yadA* transcription results from the direct binding of OmpR to the *yadA* promoter region. In the case of *hemR* an indirect and negative mechanism of OmpR-dependent regulation was proposed.

The starting point for an investigation of the role of OmpR in the regulation of *kdgM2* expression was a comparative genomic analysis that revealed the presence of clusters of genes, characteristic for phytopathogens, that encode proteins involved in the uptake and catabolism of pectin derivatives, including OM proteins KdgM2 and KdgM1, and transcriptional regulator KdgR. Furthermore, bioinformatic analysis of the *Y. enterocolitica* genome revealed the lack of extracellular pectinases typical of plant pathogens, which was confirmed by *in vitro* and *in vivo* studies.

In the next part of this research project, experiments using deletion mutants, reporter gene fusions, RT-qPCR, SDS-PAGE and EMSA demonstrated the involvement of OmpR in the reciprocal regulation of KdgM1 and KdgM2, as a result of negative regulation of *kdgM2* transcription and a positive regulatory effect on *kdgM1*. KdgR has been established as the repressor of the pectinolysis pathway genes and OmpR as a regulator influencing the expression of *kdgM2* and *kdgM1* directly, and also indirectly by repressing *kdgR*.

In addition, the important role of KdgM2 in the modulation of OM permeability in *Y. enterocolitica* was confirmed.

In summary, this body of research represents the first comprehensive characterization of the role of the response regulator OmpR in modulating the OM proteome composition of *Y. enterocolitica*. This work has revealed novel members of the *Y. enterocolitica* OmpR regulon and has shown that OmpR, through positive or negative regulation of their transcription, plays an important adaptive role. Taken together, these findings highlight OmpR as the integrator of several cellular processes regulating the dual saprophytic and pathogenic lifestyles of *Y. enterocolitica*.

1. Wstęp

Yersinia enterocolitica to gramujemna, chorobotwórcza pałeczka, czynnik etiologiczny jersiniozy – ostrej lub przewlekłej, odzwierzęcej choroby zakaźnej, która może przyjmować różne postaci kliniczne, najczęściej żołądkowo-jelitowe. Jak wynika z raportu EFSA i ECDC tylko w roku 2015, na terenie Europy, potwierdzono 7202 przypadków jersiniozy, co czyni ją trzecią najczęściej zgłaszaną zoonozą w UE [1]. *Y. enterocolitica* jest heterogennym gatunkiem obejmującym sześć biotypów, które różnią się pod względem patogenności. Szczepy o niskiej zjadliwości należą do biotypu 2-5, natomiast wysoce chorobotwórcze, najbardziej niebezpieczne dla człowieka szczepy zaklasyfikowano do biotypu 1B [2]. *Y. enterocolitica* zdolna jest do kolonizacji różnych nisz ekologicznych w obrębie organizmu gospodarza, jak i poza nim – w środowisku naturalnym. Bakterie tego gatunku są powszechnie izolowane z wody i gleby, a ich naturalnym rezerwuarem są zwierzęta, w szczególności trzoda chlewna. Do zakażenia pałeczkami jersinia dochodzi głównie w następstwie spożycia produktów pochodzenia zwierzęcego i roślinnego, zanieczyszczonych odchodami zwierząt. Głównym źródłem infekcji jest niedogotowana wieprzowina i nieumyte warzywa, w tym sałata [3, 4]. *Y. enterocolitica* to enteropatogen, który dzięki syntezie ureazy [5] jest w stanie przeżyć w kwaśnym środowisku żołądka i dotrzeć do jelita krętego, gdzie rozpoczyna się pierwszy etap patogenezy, tj. inwazja tkanki nabłonkowej. Bakterie w wyniku inwazji komórek M nabłonka limfoidalnego jelit, docierają do grudek limfatycznych (kępek Peyera), skąd mogą przedostać się z limfą do krezkowych węzłów chłonnych. Namnażanie się bakterii w tkance limfatycznej prowadzi do rozwinięcia się stanu zapalnego. Możliwa jest także infekcja wątroby lub śledziony, a nawet infekcja systemowa [2, 6].

Y. enterocolitica syntetyzuje wiele czynników wirulencji związanych z błoną zewnętrzną (OM), które biorą udział w adhezji i inwazji nabłonka jelita i/lub pozwalają skolonizować tkanki obwodowe [2, 7]. Do nich należy: (i) białko YadA, pełniące funkcję adhezyny i warunkujące oporność *Y. enterocolitica* na bakteriobójcze działanie surowicy; (ii) inwazyna Inv, odpowiedzialna za aktywne wnikanie bakterii do komórek tkanki nabłonkowej jelita; (iii) adhezyna Ail, istotna w oporności na bakteriobójczą aktywność surowicy; (iv) adhezyjne fimbrie Myf [8, 9]. Do pełnej zjadliwości *Y. enterocolitica* konieczna jest aktywność systemu sekrecji III typu Ysc-Yop. Na system ten składa się aparat sekrecyjny Ysc, który odpowiada za sekrecję i translokację białek Yop do komórki eukariotycznej. Białka Yop, to główne czynniki wirulencji, które hamują nieswoistą odpowiedź immunologiczną gospodarza, blokując produkcję cytokin prozapalnych,

zaburzając ścieżki przekazywania sygnału oraz prawidłową organizację cytoszkieletu makrofagów i neutrofili, a także indukując ich apoptozę [10-12].

Pozyskiwanie żelaza i systemy jego magazynowania odgrywają również istotną rolę w fizjologii i zjadliwości *Y. enterocolitica*, umożliwiając bakteriom adaptację do określonych nisz, na zewnątrz oraz wewnątrz organizmu gospodarza, gdzie dostęp do żelaza jest ograniczony [13, 14]. W warunkach niedoboru żelaza *Y. enterocolitica* reaguje derepresją różnych systemów pobierania tego pierwiastka [15]. Do nich należą systemy transportu Fe^{3+}-sideroforów i hemu, w których kluczową rolę pełnią specyficzne receptory błony zewnętrznej [15, 16].

Przegląd sekwencji białkowych zdeponowanych w bazie UniProt, a także dane literaturowe wskazują, że oprócz wyżej wymienionych czynników wirulencji, do białek błony zewnętrznej *Y. enterocolitica* należą również białka typowe dla bakterii gramujemnych, czyli lipoproteiny i białka o strukturze β-baryłki, w tym m.in. poryny dyfuzji ogólnej, białka tworzące kanały specyficzne substratowo, białka wchodzące w skład kompleksów pomp *efflux* oraz kompleksu Bam (ang. β-barrel-assembly machinery).

Wiele właściwości fizjologicznych komórki *Y. enterocolitica*, zależy od czynników środowiskowych. Charakterystyczna dla *Y. enterocolitica* jest termoregulacja syntezy rzęsek, gładkiego lipopolisacharydu (LPS) czy czynników wirulencji [15, 17]. Bakterie dostosowując się do zmian pH, osmolarności i temperatury, dostępności składników odżywczych/jonów, a także broniąc się przed aktywnością substancji toksycznych, mogą przeżyć w określonych niszach ekologicznych oraz skutecznie konkurować z naturalnym mikrobiomem [6, 18].

Sygnały środowiskowe są odbierane i przekształcane w komórkową odpowiedź adaptacyjną przez złożone systemy regulatorowe, w tym dwuskładnikowe systemy transdukcji sygnału (TCS, ang. two-component system) [19, 20]. Systemy TCS występują powszechnie zarówno w bakteriach saprofitycznych jak i patogennych, a archetypem jest system EnvZ/OmpR niepatogennej *Escherichia coli* K-12, zidentyfikowany podczas badania osmoregulacji ekspresji poryn OmpC i OmpF [20, 21]. Mechanizm transdukcji sygnału z udziałem pary białek, EnvZ i OmpR jest dobrze poznany [22-25]. Białko sensorowe EnvZ jest kinazą histydynową zlokalizowaną w błonie cytoplazmatycznej, która odbierając sygnał środowiskowy przez domenę sensorową, podlega autofosforylacji. Następnie grupa fosforanowa z reszty histydyny domeny centralnej EnvZ zostaje przeniesiona na resztę kwasu asparaginowego N-końcowej domeny regulatorowej białka OmpR. Fosforylacja regulatora OmpR prowadzi do zmian konformacyjnych w obrębie C-końcowej domeny efektorowej i do związania się OmpR-P do DNA. Miejsce interakcji z sekwencjami regulatorowymi

w regionie promotorowym oraz z polimerazą RNA determinuje pozytywną lub negatywną regulację transkrypcji genów. Regulator OmpR poza fosforylacją przez partnerską kinazę EnvZ, może być aktywowany przez małe fosfodonory jak np. fosforan acetylu [22, 26]. Co więcej, dowiedziono, że białko OmpR może wiązać się do DNA bez fosforylacji [22], chociaż należy podkreślić, że aktywacja poprzez dołączenie grupy fosforanowej zwiększa powinowactwo wiązania i może zmienić specyficzność [23]. Ponadto, wyniki ostatnich badań nad systemem EnvZ/OmpR *Salmonella* wskazują, że wiązanie OmpR do regionów regulatorowych genów może wynikać nie tylko z fosforylacji przez EnvZ, ale także być efektem zmian w topologii DNA, co pozwalałoby OmpR oddziaływać z sekwencjami docelowymi i wpływać na transkrypcję również poprzez modulowanie struktury DNA [27, 28]. W oparciu o analizę sekwencji DNA, do której przyłącza się białko OmpR w regionie promotorowym genu *ompC* i *ompF E. coli,* ustalono sekwencję *consensus* [TTTTACTTTTTG(A/T)AACATAT] o wielkości 20 pz, bogatą w AT i charakteryzującą się obecnością motywu centralnego G(A/T)AAC [29-31]. Sekwencja ta posłużyła do zidentyfikowania kilku OmpR-zależnych genów u bakterii z rodziny Enterobacteriaceae. Do motywów DNA zaproponowanych jako te, które warunkują wiązanie regulatora OmpR należą również CATnT [32], GTnTCA [28] i TnTnnC [33]. Ponadto, wykazano, że OmpR może regulować transkrypcję genów wiążąc się z sekwencją DNA o niskim stopniu podobieństwa do *consensus* [34] co niezmiernie utrudnia poszukiwania genów regulonu OmpR na podstawie analiz *in silico.*

Funkcja białka OmpR u *E. coli* K-12 jest od lat przedmiotem intensywnych badań, które wskazują, że oprócz regulacji genów kodujących poryny dyfuzji ogólnej OmpC i OmpF regulator ten bierze udział w kontroli wielu procesów fizjologicznych, w tym uczestniczy m.in. w regulacji biosyntezy rzęski [26], w transporcie kwasów tłuszczowych [35], czy w procesie morfogenezy [36]. Badania rozszerzone na bakterie chorobotwórcze dla ludzi i zwierząt wykazały, że system EnvZ/OmpR patogenów uczestniczy w regulacji ekspresji zarówno genów metabolizmu podstawowego jak i wirulencji, w odpowiedzi na zmiany osmolarności lub pH. Dla *Shigella flexneri* stwierdzono OmpR-zależną ekspresję genów *vir,* które są odpowiedzialne za inwazję komórek nabłonka [37]. W uropatogennej *E. coli,* system EnvZ/OmpR zaangażowany jest w regulację syntezy adhezyjnych fimbrii typu I [38, 39]. Ponadto białko OmpR *Salmonella* jest dobrze scharakteryzowanym aktywatorem systemu dwuskładnikowego SsrA/SsrB (kodowanego w obrębie wyspy patogenności SPI-2) i czynnika transkrypcyjnego HilD (kodowanego w SPI-1) przez co warunkuje zdolność do patogenezy [27].

Wieloletnie badania prowadzone w grupie badawczej prof. Katarzyny Brzostek dowiodły, że system EnvZ/OmpR pełni ważną funkcję regulatorową u *Y. enterocolitica* (bioserotyp 2/O:9). Do identyfikacji celów działania systemu EnvZ/OmpR wykorzystano klasyczne podejście opierające się na badaniu fizjologicznych konsekwencji utraty białka OmpR [40] czy metodę mutagenezy transpozonowej, polegającą na analizie funkcjonalnej komplementacji puli mutantów transpozonowych Tn*5*-*lacZ* w szczepie Δ*ompR* [41]. Udowodniono m.in., że EnvZ/OmpR kontroluje namnażanie się bakterii w komórkach makrofagów [42], moduluje ekspresję inwazyny [43] i wpływa na ruchliwość *Y. enterocolitica* przez pozytywną regulację ekspresji operonu *flhDC* kodującego aktywator genów regulonu rzęskowego [44]. Ponadto, wykazano, że istnieje korelacja między wrażliwością *Y. enterocolitica* na bakteriobójcze działanie surowicy ludzkiej a aktywnością OmpR [45]. Wyniki tych badań sugerują również, że za to zjawisko mogą być odpowiedzialne OmpR-zależne ilościowe/jakościowe zmiany w składzie białek błony zewnętrznej.

W oparciu o zebrane dotychczas wyniki wysunięto hipotezę, według której białko OmpR jako globalny regulator ekspresji genów może wpływać na właściwości wirulentne oraz adaptacyjne *Y. enterocolitica*, poprzez modulowanie składu białkowego błony zewnętrznej.

2. Cel pracy

Celem niniejszej rozprawy doktorskiej było poznanie funkcji białka OmpR w modulowaniu proteomu błony zewnętrznej *Y. enterocolitica* (bioserotyp 2/O:9), w tym:

- analiza wpływu regulatora OmpR na poziom białek błony zewnętrznej *Y. enterocolitica* metodą różnicowej analizy proteomicznej typu *shotgun*;
- identyfikacja genów regulonu OmpR oraz wyjaśnienie roli OmpR w regulacji ich ekspresji na poziomie transkrypcyjnym.

3. Omówienie wyników

3.1. Analiza wpływu regulatora OmpR na poziom białek błony zewnętrznej *Y. enterocolitica* metodą różnicowej analizy proteomicznej typu *shotgun*

Publikacja 1: Nieckarz i wsp. (2016) Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR. Environ Microbiol 18: 997–1021.

Kompleksowe spojrzenie na funkcje regulatora OmpR u bakterii jest obecnie możliwe dzięki zastosowaniu nowoczesnych technik „omicznych" (transkryptomicznych i proteomicznych). Do identyfikacji OmpR-zależnych genów u *E. coli* oraz *Salmonella* wykorzystano metodę wysokoprzepustowej analizy transkryptomicznej, z zastosowaniem mikromacierzy DNA [46] i sekwencjonowania RNA (RNA-seq) [34], a także metodę immunoprecypitacji „chromatyny bakteryjnej" (ChIP-on-chip, ChIP-seq) [28, 34] oraz technikę SELEX (ang. systematic evolution of ligands by exponential enrichment) [33]. Do identyfikacji białek, których poziom w komórce zależy od aktywności białka OmpR, zastosowano dotychczas „proteomikę żelową", która polega na kombinacji elektroforezy dwukierunkowej z metodą spektrometrii mas typu MALDI-ToF (ang. matrix assisted laser desorption ionisation-time of flight mass spectrometry) [47, 48].

W badaniach, będących podstawą niniejszej rozprawy doktorskiej, do identyfikacji białek błony zewnętrznej *Y. enterocolitica*, regulowanych przez białko OmpR zastosowano różnicową analizę proteomiczną metodą *shotgun*. Strategia ta polegała na trawieniu trypsyną frakcji białek błonowych, wzbogaconych o białka błony zewnętrznej i analizie mieszanin peptydów w systemie HPLC sprzężonym ze spektrometrem mas Orbitrap Velos. Tandemowy spektrometr mas umożliwił zastosowanie strategii *high-high*, w której pomiary widm masowych w trybach MS i MS/MS charakteryzują się wysoką rozdzielczością.

W pierwszym etapie prac dokonano optymalizacji protokołu izolacji białek osłon komórkowych wzbogaconych o białka OM. Podczas dopracowywania metody wykorzystano szereg detergentów w zmiennych kombinacjach i stężeniach, jak np. N-laurylosarkozynian sodu, deoksycholan sodu, Invitrosol, CHAPS czy SDS oraz modyfikowano warunki trawienia trypsyną mieszanin białek w roztworze lub w kolumienkach ultrafiltracyjnych (metoda FASP, ang. filter aided sample preparation). Analizy LC-MS/MS różnych frakcji, uzyskiwanych podczas testowania protokołów izolacji białek OM, pozwoliły na wybór optymalnej metodyki, którą zastosowano we właściwych eksperymentach. Wzbogacenie frakcji nierozpuszczalnej w sarkozylu o białka OM, o silnych właściwościach hydrofobowych,

dokonano z użyciem SDS-u i deoksycholanu sodu. Analizy MS przeprowadzono w Środowiskowym Laboratorium Spektrometrii Mas w Zakładzie Biofizyki IBB PAN.

Różnicowa analiza proteomiczna polegała na jakościowej i ilościowej analizie danych frakcji białkowych szczepów różniących się obecnością białka OmpR, tj. dzikiego szczepu Ye9 i izogenicznego mutanta Δ*ompR* (szczep AR4) [42] oraz hodowanych w różnych warunkach temperatury (26°C vs 37°C), osmolarności (86 mM NaCl vs 386 mM NaCl) i pH (5 vs 7).

Do analizy jakościowej MS zastosowano oprogramowanie MASCOT i MScan, do analizy ilościowej MSparky i MSconvert. Analizę statystyczną prowadzącą do utworzenia list białek różnicowych (tzn. białek, których poziom różnił się pomiędzy porównywanymi wariantami) uzyskano dzięki programowi Diffprot. Jako kryterium istotności przyjęto wartość $q \leq 0{,}05$, a jako parametr krotności zmiany (ang. fold change) wartość $\geq 1{,}5$.

Pierwszym etapem różnicowej analizy proteomicznej, było porównanie jakościowe i ilościowe białek OM szczepu dzikiego Ye9 *Y. enterocolitica*, hodowanego w różnych warunkach osmolarności, pH i temperatury. Do białek, których poziom wzrastał w temperaturze 37°C należała adhezyna YadA oraz elementy systemu sekrecji Ysc-Yop zgodnie z wcześniejszymi doniesieniami literaturowymi [49-51]. W warunkach wysokiej osmolarności (386 mM NaCl) zaobserwowano zwiększoną syntezę białka OmpC potwierdzając tym samym osmoregulację tej poryny u *Y. enterocolitica* [52]. Indukcję niskim pH (5,0) stwierdzono w przypadku podjednostek ureazy UreA i UreG [47] oraz białka MyfC zaangażowanego w kotwiczenie fimbrii Myf, zgodnie z wcześniejszymi badaniami [53]. Otrzymane wyniki potwierdziły wpływ badanych fizyko-chemicznych parametrów środowiskowych na składniki proteomu osłon *Y. enterocolitica.*

Różnicowa analiza frakcji białek szczepu dzikiego i mutanta Δ*ompR* pozwoliła zidentyfikować łącznie 120 białek osłon, w tym związanych pośrednio lub bezpośrednio z OM, których poziom zależał od obecności OmpR, w określonych warunkach środowiska. Białka regulowane przez OmpR (pozytywnie lub negatywnie) podzielono, zgodnie z procesami biologicznymi, w których uczestniczą, na kilka kategorii Gene Ontology (GO). Wykazano, że około jedna trzecia OmpR-zależnych białek uczestniczy w dyfuzji oraz w aktywnym transporcie przez błony. Wśród tych białek znajdują się poryny dyfuzji ogólnej OmpC i OmpF. Regulacja ekspresji genów *ompC* i *ompF* w zależności od OmpR została już wcześniej udowodniona u *Y. enterocolitica* [40], *E. coli* [21] oraz *Y. pestis* [54]. Panel poryn zależnych od OmpR obejmuje także anionowo-specyficzną porynę PhoE [55], porynę specyficzną wobec sacharozy ScrY [56], porynę OmpW, która może być

zaangażowana w odpowiedź na różne czynniki stresowe [57], a także porynę OmpX, o niezdefiniowanej funkcji, zgodnie z wcześniejszymi danymi uzyskanymi dla *Y. enterocolitica* [45] i *Y. pestis* [58].

Największe ilościowe zmiany wśród składników proteomu OM, zależne od regulatora OmpR, dotyczyły białka KdgM2, homologa poryn KdgM i KdgN specyficznych dla transportu oligogalakturonianów, które zidentyfikowano u patogenu roślin *Dickeya dadantii* [59]. W warunkach 26°C, mutant *ompR* charakteryzował się ponad 100-krotnym wzrostem poziomu KdgM2, co sugerowało rolę OmpR w inhibicji ekspresji genu tej poryny.

Wśród białek, których poziom zależał od OmpR zidentyfikowano FadL, transporter OM specyficzny wobec kwasów tłuszczowych, co jest zgodne z wcześniejszymi doniesieniami mówiącymi o negatywnej, bezpośredniej regulacji transkrypcji *fadL* przez OmpR u *E. coli* [35]. Wyniki sugerują, że OmpR może hamować transport aminokwasów poprzez negatywny wpływ na syntezę transporterów aminokwasów (np. transportera seryny/treoniny SstT czy permeazy CycA uczestniczącej w transporcie D-alaniny, D-seryny i glicyny), a promować transport peptydów do wnętrza komórki, wpływając pozytywnie na poziom transporterów peptydowych TppB, OppA, OppD i OppF. Udział OmpR w pozytywnej regulacji ekspresji genu *tppB* kodującego permeazę tripeptydów TppB wykazano wcześniej dla *S.* Typhimurium [60] i *E. coli* [61]. Wpływ OmpR na poziom permeazy CycA, która warunkuje pobieranie do komórki D-alaniny, aminokwasu kluczowego w procesie strukturyzowania peptydoglikanu [62], sugeruje, że aktywność regulatora OmpR może mieć znaczenie dla metabolizmu ściany komórkowej. Różnicowana analiza proteomiczna wskazała na pozytywną OmpR-zależną regulację poziomu białka DcuA odpowiedzialnego za transport C4-dikarboksylanów, w tym bursztynianu, fumaranu i jabłczanu, które są źródłem węgla i energii [63], co świadczy o zaangażowaniu regulatora OmpR w modulowanie procesów metabolicznych. Uzyskane wyniki wykazały również udział OmpR w pozytywnej regulacji ekspresji białka AcrA, które jest częścią kompleksu pompy *efflux* AcrAB-TolC, potwierdzając tym samym wyniki wcześniejszych badań naszego zespołu [41].

Druga najliczniejsza kategoria białek GO, zależnych od OmpR, jest związana z patogenezą. W przypadku większości białek w tej kategorii stwierdzono niższy poziom syntezy w szczepie dzikim w stosunku do szczepu mutanta *ompR*, z wyjątkiem białka OM MyfC zaangażowanego w kotwiczenie fimbrii Myf (w warunkach pH 5 i temperatury 37°C) oraz ureazy (w warunkach 37°C). Dużą grupę białek zależnych od OmpR i związanych z patogenezą stanowiły komponenty systemu sekrecji III typu Ysc-Yop. Analiza MS

wskazała także na wyższy poziom białka YadA w mutancie *ompR* w stosunku do szczepu dzikiego we wszystkich testowanych warunkach wzrostu.

Kolejna kategoria GO obejmuje białka zaangażowane w organizację błony zewnętrznej, tj. białka kompleksu Bam odpowiedzialnego za fałdowanie białek OM do konformacji β-baryłki [64] oraz białka WbcV, WbcU i WbcT, które uczestniczą w syntezie unikatowego dla *Y. enterocolitica* serotypu O:9, O-swoistego polisacharydu wchodzącego w skład cząsteczki LPS [65].

Spośród białek, których poziom zależał od regulatora OmpR wyodrębniono również grupę związaną z utrzymaniem homeostazy żelaza. Białka należące do tej kategorii obejmowały m.in. trzy transportery OM, zwane także transporterami zależnymi od TonB: receptory FepA i FecA odpowiedzialne za asymilację żelaza za pośrednictwem sideroforów oraz HemR, receptor zaangażowany w wychwyt hemu/hemoprotein [16, 66]. Wykazano wyższy poziom HemR w mutancie *ompR* w stosunku do szczepu dzikiego w warunkach 37°C.

Podsumowując, dzięki zastosowaniu różnicowej analizy proteomicznej metodą *shotgun* zidentyfikowano 120 białek osłon, w tym związanych pośrednio lub bezpośrednio z błoną zewnętrzną, których poziom zależał od regulatora OmpR. Udokumentowano wpływ OmpR na szereg białek biorących udział w dyfuzji i aktywnym transporcie do wnętrza komórki jak również w procesach sekrecji czy *efflux*. Ponadto, otrzymane wyniki sugerują udział OmpR w regulacji wirulencji *Y. enterocolitica* poprzez modulowanie poziomu syntezy białek umożliwiających przeżycie w warunkach kwasowego pH, systemu sekrecji III typu Ysc-Yop czy białka YadA warunkującego adhezję i oporność komórki na bakteriobójcze działanie surowicy.

Zdefiniowany w tej pracy OmpR-zależny proteom błonowy stanowił punkt wyjścia do szczegółowych badań genetycznych i fizjologicznych opisujących nowe cele działania regulatora OmpR.

3.2. Identyfikacja genów regulonu OmpR oraz wyjaśnienie roli OmpR w regulacji ich ekspresji na poziomie transkrypcyjnym

Publikacja 1: Nieckarz i wsp. (2016) Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR. Environ Microbiol 18: 997–1021.

Publikacja 2: Nieckarz i wsp. (2017) The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*. Front Cell Infect Microbiol 7: 366.

Kolejnym celem mojej dysertacji oraz naturalną konsekwencją przeprowadzonych analiz proteomicznych była identyfikacja genów należących do regulonu OmpR i kodujących białka ważne w fizjologii *Y. enterocolitica*. Punktem wyjścia do identyfikacji genów regulonu OmpR były analizy *in silico* w celu wskazania miejsc wiązania OmpR w regionach promotorowych genów kodujących zidentyfikowane białka różnicowe. Do poszukiwania miejsc wiązania OmpR zastosowano sekwencję *consensus E. coli* [29]. Potencjalne sekwencje wiązania białka OmpR zidentyfikowano dla genów *cycA*, *dcuA*, *fecA*, *fepA*, *hemR*, *kdgM2*, *myfC*, *ompW*, *scrY* i *yadA* oraz zgodnie z wcześniejszymi doniesieniami literaturowymi, w obrębie sekwencji regulatorowych *acrA*, *fadL*, *ompC*, *ompF*, *ompX*, *tppB* [35, 41, 45, 58, 60, 61].

Do szczegółowych badań genetycznych mających na celu poznanie mechanizmu OmpR-zależnej regulacji wytypowano trzy białka OM: adhezynę YadA, receptor hemu/hemoprotein HemR oraz porynę KdgM2, dla których analiza MS wskazała wyższy poziom w mutancie *ompR Y. enterocolitica* w stosunku do szczepu dzikiego.

YadA jest homotrimerycznym białkiem, kodowanym przez gen zlokalizowany w plazmidzie wirulencji pYV. Białko YadA pełni kluczową rolę w procesie adhezji *Y. enterocolitica* do komórek eukariotycznych i do składników macierzy zewnątrzkomórkowej (kolagenu, fibronektyny i lamininy) oraz chroni bakterie przed bakteriobójczym działaniem białek układu dopełniacza wiążąc regulator C4BP i czynnik H, co m.in. blokuje opsonizację oraz powstawanie kompleksu atakującego błonę [8, 67]. Ekspresja *yadA* jest indukowana w temperaturze 37°C, co wynika ze zmiany topologii DNA w tych warunkach. Postuluje się także, że obserwowana termoregulacja ekspresji *yadA* jest koordynowana przez dwa regulatory: regulator transkrypcji VirF oraz białko histonopodobne YmoA. Przy czym VirF pozytywnie, a YmoA negatywnie reguluje syntezę YadA [50].

Punktem wyjścia do analiz genetycznych nad rolą OmpR w regulacji poziomu YadA były wyniki badań Western blot, które przeprowadzono z zastosowaniem przeciwciał specyficznych dla YadA. Wyniki tych analiz wskazały na negatywny charakter regulacji z udziałem OmpR i tym samym potwierdziły dane proteomiczne. W celu wyjaśnienia wpływu OmpR na syntezę YadA analizowano ekspresję genu *yadA,* w fuzji z *gfp,* w szczepach różniących się poziomem regulatora OmpR. Pomiary fluorescencji, metodą cytometrii przepływowej, wykazały wyższy poziom ekspresji *yadA-gfp* w mutancie *ompR* w stosunku do szczepu dzikiego, w różnych warunkach temperatury (27°C vs 37°C), pH (5 vs 7) i osmolarności (86 mM NaCl vs 386 mM NaCl). Rolę OmpR w negatywnej regulacji ekspresji *yadA* potwierdził test komplementacji mutacji dzikim allelem *ompR* wprowadzonym *in trans.* Identyfikacja potencjalnego miejsca wiązania OmpR w regionie promotorowym *yadA* sugerowała udział OmpR w bezpośredniej regulacji ekspresji genu *yadA.* W celu potwierdzenia zdolności do oddziaływania OmpR z wyznaczoną sekwencją wiążącą, przeprowadzono badania opóźnienia tempa migracji kompleksów nukleoproteinowych w natywnym żelu poliakryloamidowym tzw. testy EMSA. W tym celu oczyszczone do homogenności białko OmpR fosforylowano *in vitro* i inkubowano z odpowiednimi fragmentami DNA. Testy EMSA przeprowadzono dla sekwencji z wyznaczonym miejscem wiązania i z dwiema sekwencjami okalającymi motyw. Dzięki analizom udało się potwierdzić zdolność białka OmpR do specyficznego wiązania się z fragmentem DNA zawierającym wytypowaną sekwencję i brak reakcji w przypadku dwóch pozostałych fragmentów. Podsumowując, przeprowadzone eksperymenty udokumentowały, że białko OmpR negatywnie i bezpośrednio reguluje ekspresję genu kodującego białko YadA.

W kolejnym etapie pracy skoncentrowano się na poznaniu funkcji białka OmpR w regulacji ekspresji genu kodującego białko HemR – receptor hemu i hemoprotein u *Y. enterocolitica* [66, 68]. Hem jako grupa prostetyczna cytochromów i katalazy jest niezbędnym czynnikiem oddychania komórkowego, ponadto, stanowi ważne źródło żelaza w warunkach jego niedoboru w środowisku. Zarówno nadmiar hemu jak i żelaza jest toksyczny dla bakterii [66, 69]. W utrzymaniu homeostazy żelazowej uczestniczy białko Fur, regulator kontrolujący ekspresję wielu genów związanych z transportem żelaza/hemu u bakterii. Białko Fur pełni funkcję represora, który w kompleksie z jonami żelaza (w warunkach wysokiego stężenia Fe^{2+} w komórce) wiąże się do sekwencji promotorowej genów hamując ich transkrypcję [70].

W pierwszej kolejności, aby potwierdzić wyniki uzyskane w eksperymencie proteomicznym, określono poziom receptora HemR metodą Western blot z zastosowaniem

przeciwciał specyficznych dla HemR. Analiza immunodetekcji wykazała wyższy poziom białka HemR w mutancie *ompR* w porównaniu do szczepu dzikiego, w warunkach nadmiaru jak i niedoboru jonów żelaza. Aby ocenić rolę białka OmpR w regulacji *hemR* u *Y. enterocolitica* skonstruowano szczepy niosące fuzję transkrypcyjną *hemR-lacZYA'.* Wyższy poziom aktywności promotora *hemR* w mutancie *ompR,* w stosunku do szczepu dzikiego, wskazywał na negatywną rolę biała OmpR w regulacji transkrypcji *hemR*. Zaobserwowana zwiększona ekspresja *hemR* w warunkach głodu żelazowego potwierdzała, że *hemR* znajduje się pod kontrolą Fur. Ponadto, w tych samych warunkach zanotowano zniesienie OmpR-zależnej regulacji, co sugerowało, że OmpR może kontrolować ekspresję *hemR* w sposób zależny od Fur. W teście EMSA nie wykazano specyficznego oddziaływania białka OmpR z potencjalnym miejscem wiązania regulatora zidentyfikowanym *in silico,* w obszarze regulatorowym *hemR*. Podsumowując, wynik analizy EMSA oraz badania aktywności promotora *hemR* sugerowały pośredni, negatywny mechanizm regulacji *hemR* – być może poprzez pozytywny wpływ na ekspresję *fur*. Zagadnienie to jest przedmiotem prowadzonych obecnie badań.

Proteomiczna analiza różnicowa wykazała, że największe ilościowe zmiany w składzie proteomu *Y. enterocolitica,* wynikające z braku regulatora OmpR, dotyczą białka KdgM2. Białko KdgM2 *Y. enterocolitica*, na podstawie podobieństwa sekwencji aminokwasowej, zaklasyfikowano do rodziny białek KdgM, pierwotnie zidentyfikowanych u patogenu roślin *D. dadantii* (dawniej *Erwinia chrysanthemi*). Rodzina białek KdgM obejmuje monomeryczne poryny, które uczestniczą w transporcie oligogalakturonianów (OGA) – produktów degradacji pektyn [59, 71].

D. dadantii podobnie jak inne bakterie należące do rodzaju *Dickeya* i *Pectobacterium,* wytwarza szerokie spektrum enzymów pektynolitycznych o różnej lokalizacji (głównie zewnątrzkomórkowej, ale i peryplazmatycznej lub cytoplazmatycznej) oraz o odmiennych właściwościach katalitycznych, co umożliwia skuteczną degradację pektyn obecnych w ścianach komórek roślinnych, prowadząc do maceracji tkanek i choroby rośliny.

Szlak pektynolizy został bardzo dobrze poznany u *D. dadantii* [59, 72]. Dzięki zewnątrzkomórkowym enzymom pektynolitycznym (o aktywności liazy pektynowej, poligalakturonazy, metyloesterazy i acetyloesterazy), wydzielanym przez system sekrecji typu II *Out,* dochodzi do deestryfikacji i depolimeryzacji pektyn do oligogalakturonianów, które przedostają się do przestrzeni peryplazmatycznej dzięki specyficznym porynom błony zewnętrznej KdgM i KdgN. W peryplazmie OGA są degradowane dalej do krótszych oligogalakturonianów (digalakturonianów-tetragalakturonianów), które są transportowane

do cytoplazmy dzięki obecnemu w błonie cytoplazmatycznej systemowi transportu z rodziny ABC – TogMNAB i symporterowi TogT. Jednym z końcowych produktów degradacji OGA jest 2-keto-3-deoksyglukonian (KDG). KDG wiąże się do represora KdgR co stymuluje odłączenie się białka od sekwencji regulatorowych genów/operonów szlaku pektynolizy i indukuje ich ekspresję. W wyniku wzrostu *D. dadantii* w podłożu z OGA dochodzi do derepresji genów regulonu KdgR. Końcowe produkty katabolizmu pektyn – pirogronian i aldehyd 3-fosfoglicerynowy są włączane do cyklu kwasu cytrynowego stając się źródłem energii dla komórki bakteryjnej [73-75].

Analizy genomowe wykazały obecność niekompletnego szlaku degradacji pektyn u niektórych bakterii z rodziny Enterobacteriaceae, w tym u *Klebsiella pneumoniae, S. enterica* sv Typhimurium, a także *Y. enterocolitica*. Jak wskazują analizy bioinformatyczne *Y. enterocolitica* nie syntezuje zewnątrzkomórkowych enzymów pektynolitycznych, a także systemu transportu *Out*, który jest niezbędny do ich sekrecji [76]. W efekcie pałeczki *Yersinia* nie są zdolne do maceracji tkanek roślinnych, co wykazano w trakcie badań, prowadzonych w ramach tej rozprawy doktorskiej.

Punktem wyjścia do prac nad określeniem funkcji białka OmpR, w regulacji poziomu syntezy KdgM2 była analiza bioinformatyczna genów/operonów związanych z transportem i depolimeryzacją OGA u *Y. enterocolitica*. Wyniki tych analiz pozwoliły na identyfikację dwóch genów, *kdgM2* i *kdgM1*, kodujących odpowiednio poryny KdgM2 i KdgM1. Przeprowadzona analiza RT-PCR wykazała, że *kdgM2* razem z *pelP* i *sghX*, kodującymi peryplazmatyczną liazę pektynową PelP oraz białko wiążące i akumulujące oligogalakturoniany SghX, tworzą operon. Wyróżniono również gen *pehX* peryplazmatycznej egzopoligalakturonazy PehX oraz operon *pelW-togMNAB* kodujący cytoplazmatyczną liazę pektynową PelW i transporter błony cytoplazmatycznej TogMNAB. Ponadto zidentyfikowano gen *kdgR* kodujący represor KdgR genów/operonów związanych z pobieraniem i katabolizmem OGA, zlokalizowany w sąsiedztwie genu *ogl* kodującego cytoplazmatyczną liazę oligogalakturonianu *Ye*OGL.

Badania nad rolą OmpR w regulacji poziomu KdgM2 rozpoczęto porównaniem profilu białkowego błony zewnętrznej metodą SDS-PAGE i identyfikacją wybranych białek metodą spektrometrii mas. Do analizy wykorzystano mutanty *Y. enterocolitica* z delecją genu *kdgM2*, skonstruowane metodą rekombinacji homologicznej w szczepie dzikim (*kdgM2*) oraz w mutancie *ompR* (*ompRkdgM2*). W celu derepresji genów białek KdgM2 i KdgM1, a także innych genów regulonu KdgR hodowle *Y. enterocolitica* prowadzono w obecności OGA. Dodatkowo do analiz zastosowano mutanta *Y. enterocolitica* z delecją genu *kdgR*

oraz podwójnego mutanta *ompRkdgR*. Analiza profili białkowych dowiodła, że KdgR i OmpR hamują ekspresję białka KdgM2. Ponadto addytywny efekt mutacji *kdgR* i *ompR*, sugerował, że KdgR i OmpR mogą regulować ekspresję *kdgM2* niezależnie.

Aby scharakteryzować molekularny mechanizm OmpR-zależnej regulacji *kdgM2* zastosowano fuzję translacyjną *kdgM2* z genem reporterowym *rfp*. Analiza fluorescencji w szczepach różniących się poziomem OmpR oraz w warunkach derepresji (w obecności OGA) potwierdziła negatywny wpływ OmpR i KdgR na ekspresję *kdgM2*. Ponieważ *kdgM2* razem z *pelP* i *sghX*, tworzą operon postanowiono zbadać wpływ białka OmpR na poziom mRNA *kdgM2* i *pelP* metodą RT-qPCR. Wyniki tej analizy potwierdziły rolę OmpR w negatywnej regulacji *kdgM2* i *pelP*. Test pektynolityczny, pozwalający na półilościową ocenę poziomu liazy pektynowej PelP, wykazał zwiększoną aktywność enzymu w mutancie *ompR*, *kdgR* jak i w podwójnym mutancie *ompRkdgR*, potwierdzając tym samym funkcję obu białek w regulacji ekspresji operonu *kdgM2-pelP-sghX*.

Do analizy roli OmpR w regulacji ekspresji genu *kdgM1*, kodującego drugą porynę specyficzną dla OGA (KdgM1), zastosowano szczepy niosące chromosomową fuzję transkrypcyjną *kdgM1-lacZYA'*. Badania aktywności promotora *kdgM1* wykazały, że KdgR jest represorem a OmpR pełni funkcję pozytywnego regulatora tego genu.

W kolejnym etapie prac badano wpływ OmpR na ekspresję genu represora KdgR. Analizy aktywności promotora *kdgR* w fuzji transkrypcyjnej z genem reporterowym *lacZ* wykazały negatywny wpływ OmpR na ekspresję *kdgR*. Wynik ten potwierdzono analizując poziom mRNA *kdgR* metodą RT-qPCR.

Dzięki analizom *in silico* wyznaczono sekwencje wiązania KdgR w obszarze promotorowym wszystkich zidentyfikowanych genów/operonów szlaku depolimeryzacji OGA, a także potencjalne miejsca wiązania OmpR w obszarze regulatorowym operonu *kdgM2-pelP-sghX* oraz genów *kdgM1* i *kdgR*. Wyznaczona w obrębie sekwencji promotorowej *kdgM1* sekwencja wiążąca OmpR pokrywa się z miejscem wiązania dla represora KdgR co sugeruje, że OmpR pozytywnie reguluje ekspresję *kdgM1* pełniąc funkcję antyrepresora. Testy EMSA wykazały, że OmpR specyficznie wiąże się z obszarem promotorowym *kdgM1*, *kdgM2-pelP-sghX* oraz *kdgR* co świadczy o bezpośredniej regulacji ekspresji wymienionych genów/operonów.

W celu wyjaśnienia czy OmpR-zależna regulacja KdgR może wpływać na ekspresję innych genów regulonu KdgR *Y. enterocolitica* postanowiono zbadać aktywność promotora genu *pehX*, oraz operonu *pelW-togMNAB* w fuzji transkrypcyjnej z genem reporterowym *lacZ*. Wyniki sugerowały pozytywną OmpR-zależną regulację ekspresji *pehX* i

pelW-togMNAB. Ponieważ, w obrębie sekwencji regulatorowych *pehX* i *pelW-togMNAB* nie zidentyfikowano potencjalnych sekwencji wiążących OmpR wynik ten świadczy o pośrednim mechanizmie regulacji, w wyniku hamowania ekspresji *kdgR*.

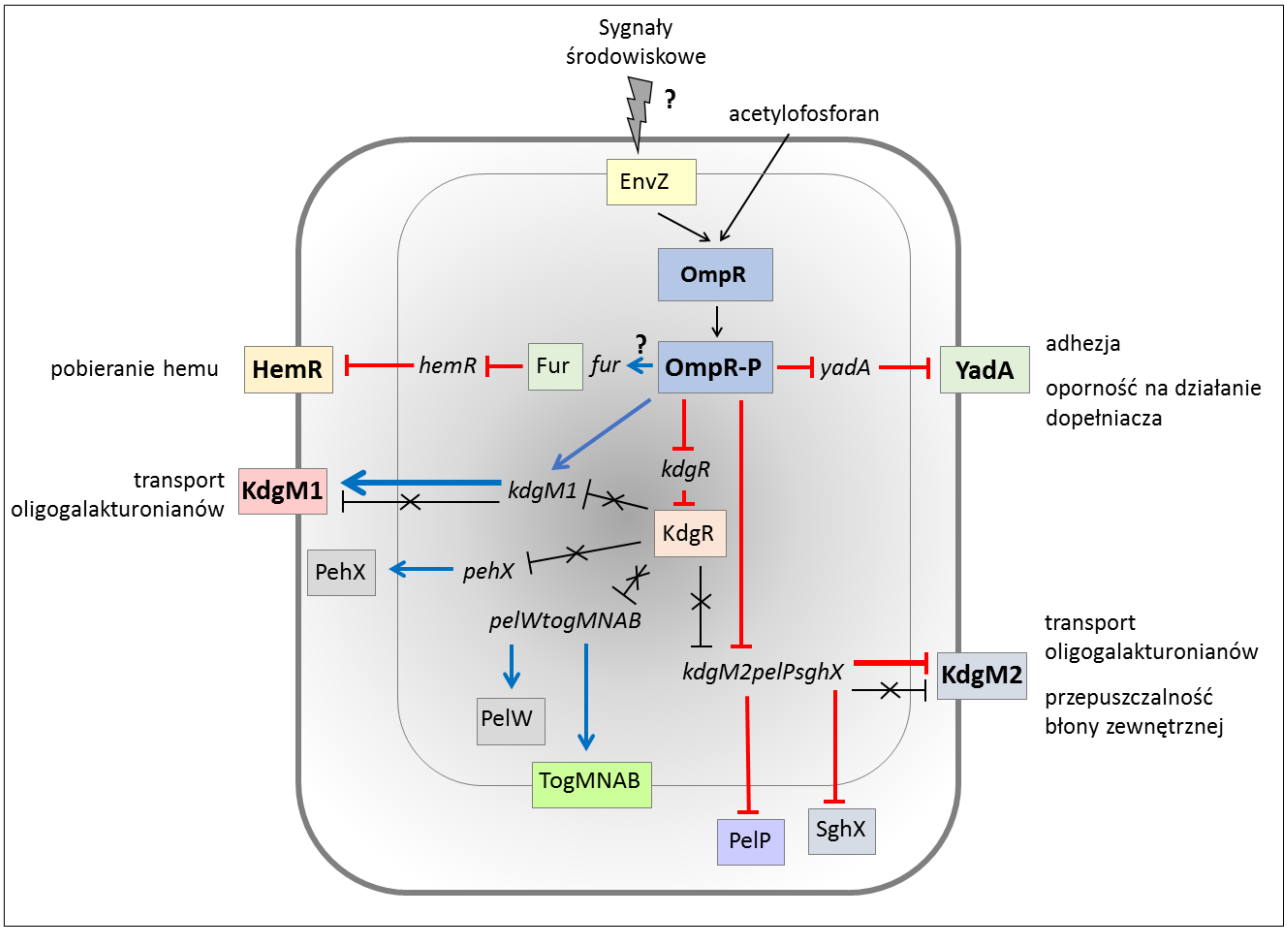
W toku kolejnych badań skoncentrowano się na poznaniu biologicznej funkcji białka KdgM2 i weryfikacji wysuniętej hipotezy o roli poryny KdgM2 w transporcie innych związków do komórki *Y. enterocolitica* oraz w regulacji przepuszczalności OM. Badania podjęte z wykorzystaniem szczepów różniących się poziomem białka KdgM2, w tym szczepu pozbawionego oraz nadeksprymującego KdgM2 z promotora pBAD indukowanego arabinozą, pozwoliły wnioskować o znaczeniu tego białka w przepuszczalności błony zewnętrznej *Y. enterocolitica*.

Podsumowując, badania dowiodły, że OmpR wykazuje odwrotny efekt regulatorowy na poziom syntezy poryn KdgM1 i KdgM2, w wyniku negatywnej regulacji transkrypcji *kdgM2* oraz pozytywnej *kdgM1*. Ponadto, regulator OmpR może wpływać na ekspresję *kdgM2* i *kdgM1* w sposób pośredni, poprzez modulowanie poziomu represora KdgR. Dwupoziomowa, odwrotna regulacja obu poryn może być korzystna w określonej niszy ekologicznej i poprawiać *fitness Y. enterocolitica*.

4. Podsumowanie

Analizy proteomiczne wykazały, że OmpR, w różnych warunkach środowiska, reguluje pozytywnie lub negatywnie poziom szeregu białek błony zewnętrznej pełniących w komórce *Y. enterocolitica* różnorodne funkcje. Wśród białek OmpR-zależnych zidentyfikowano znane, jak i zupełnie nowe białka, w tym także charakterystyczne dla bakterii z rodzaju *Yersinia.*

Zidentyfikowano nieznane wcześniej geny regulonu OmpR *Y. enterocolitica*, tj. *yadA, hemR, kdgM2, kdgM1* i *kdgR*, odpowiadające za proces adhezji, transport hemu, pobieranie i depolimeryzację produktów degradacji pektyn oraz przepuszczalność błony zewnętrznej. Otrzymane wyniki dowiodły, że OmpR może bezpośrednio regulować ekspresję genów poryn specyficznych dla oligogalakturonianów, jak i pośrednio, wpływając na transkrypcję genu regulatora KdgR (Ryc. 1).



Ryc. 1. Funkcja białka OmpR w modulowaniu składników proteomu błony zewnętrznej *Y. enterocolitica.* W zaproponowanym modelu, regulator OmpR fosforylowany przez acetylofosforan lub kinazę EnvZ, w odpowiedzi na sygnał środowiskowy, wiąże się do regionu promotorowego określonych genów, regulując pozytywnie (strzałki niebieskie) lub negatywnie (linie czerwone) ich transkrypcję. Efektem regulacji są zmiany w poziomie syntezy białek pełniących w komórce różnorodne funkcje.

5. Wnioski

Przedstawiona rozprawa doktorska stanowi pierwszą, kompleksową charakterystykę roli regulatora OmpR w modulowaniu proteomu błony zewnętrznej *Y. enterocolitica*.

Wyniki prezentowane w rozprawie doktorskiej znacząco poszerzyły wiedzę o roli białka OmpR w fizjologii bakterii gramujemnych i dostarczyły dowodów wskazujących na udział OmpR w regulacji właściwości wirulentnych oraz zdolności adaptacyjnych *Y. enterocolitica*.

Otrzymane wyniki sugerują, że OmpR jako globalny integrator wielu komórkowych procesów, może decydować o wyborze strategii życiowej *Y. enterocolitica*, związanej z saprofityczną lub patogenną formą życia bakterii.

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Załączniki

Publikacja oryginalna 1

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Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR

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Summary

Enteropathogenic *Yersinia enterocolitica* is able to grow within or outside the mammalian host. Previous transcriptomic studies have indicated that the regulator OmpR plays a role in the expression of hundreds of genes in enterobacteria. Here, we have examined the impact of OmpR on the production of *Y. enterocolitica* membrane proteins upon changes in temperature, osmolarity and pH. Proteomic analysis indicated that the loss of OmpR affects the production of 120 proteins, a third of which are involved in uptake/transport, including several that participate in iron or heme acquisition. A set of proteins associated with virulence was also affected. The influence of OmpR on the abundance of adhesin YadA and heme receptor HemR was examined in more detail. OmpR was found to repress YadA production and bind to the *yadA* promoter, suggesting a direct regulatory effect. In contrast, the repression of *hemR* expression by OmpR appears to be indirect. These findings provide new insights into the role of OmpR in remodelling the cell surface and the adaptation of *Y. enterocolitica* to different environmental niches, including the host.

Introduction

The enteropathogen *Yersinia enterocolitica* is a member of the genus *Yersinia*, which includes two other human pathogens: the plague bacillus *Y. pestis* and *Y. pseudotuberculosis*, a gastrointestinal pathogen (Bottone, 1997; Francis, 2013). *Yersinia enterocolitica* is a heterogeneous species classified into 60 serotypes and six biotypes that vary in pathogenicity (Thomson *et al.*, 2006). Based on genomic sequence differences, *Y. enterocolitica* has been divided into two subspecies: *enterocolitica* and *palearctica* (Neubauer *et al.*, 2000). Due to its ability to grow both outside and inside mammalian hosts, *Y. enterocolitica* experiences diverse environmental conditions. As a free-living enteric bacterium, it exhibits features that are expressed at ambient temperature, but only weakly or not at all at 37°C (mammalian body temperature), including motility, smooth lipopolysaccharide (LPS) production, invasin expression and some metabolic properties (Straley and Perry, 1995). Significantly, some of these characteristics are required in the early stages of infection of the human body (Pepe and Miller, 1993). *Yersinia enterocolitica* synthesizes numerous virulence factors that appear progressively during the process of pathogenesis and whose expression is altered in response to changes in growth conditions in the varied niches within the human body, and through the combined effects of bacterial colonization and the host response. In particular, changes in osmolarity and pH combined with temperature variation appear to have a considerable impact on the ability of Yersiniae to survive and promote successful pathogenesis (Straley and Perry, 1995). *Yersinia enterocolitica* synthesizes many virulence factors, including the outer membrane (OM) adhesins YadA, Ail, Inv and Myf, which allow the bacterial cells to adhere to and invade the intestinal epithelium and/or to colonize the peripheral tissues (Bottone, 1997; Bialas *et al.*, 2012). *Yersinia enterocolitica*

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also possesses a complex protein secretion machinery that spans both the inner and outer membranes – the Ysc Type 3 Secretion System (T3SS). This secretion apparatus enables the translocation of anti-host effector proteins known as Yops (Yersinia outer proteins) into host cells. The Ysc-Yop T3SS is required for full virulence in the late stages of the process of pathogenesis (Cornelis *et al*., 1998; Cornelis, 2002). The Yop proteins are responsible for the inhibition of host defence reactions and permit the multiplication of bacterial cells in the reticuloendothelial system (Viboud and Bliska, 2005). Iron acquisition and storage systems also play an essential role in *Y. enterocolitica* physiology and virulence by allowing the bacterium to adapt to specific niches outside and inside the human body where iron is limited (Heesemann *et al*., 1993; Perry, 1993).

Two-component regulatory systems (TCSs) constitute an important regulatory mechanism in bacterial cells that mediate a variety of adaptive responses to changes in environmental cues (Stock *et al*., 1989; Hoch and Silhavy, 1995). TCSs are found in saprophytic and pathogenic bacteria and the archetype is EnvZ/OmpR. This system was initially characterized in its role in the osmoregulation of OmpC and OmpF porin expression in non-pathogenic *Escherichia coli* K-12 (Russo and Silhavy, 1990; Hoch and Silhavy, 1995). The TCS consists of the sensor protein EnvZ, which has dual kinase/phosphatase activity, and regulatory protein OmpR, which acts to regulate transcription (Kenney, 2002). In response to environmental changes, EnvZ modulates the phosphorylation and thereby the transcriptional activity of OmpR. Besides its partner kinase EnvZ, OmpR is likely to be available for phosphorylation by small phospho-donors, e.g. acetyl-P, which may cause activation (Shin and Park, 1995; Head *et al*., 1998). Recent studies in *Salmonella* have provided evidence that the binding of OmpR to the regulatory regions of target genes may result not only from phosphorylation by EnvZ, but also as an effect of DNA relaxation in response to acid stress that allows OmpR to cooperate with the altered topology to modulate transcription (Cameron and Dorman, 2012; Quinn *et al*., 2014).

A considerable body of research indicates that OmpR is involved in the control of various cellular processes and functions in *E. coli* (Higashitani *et al*., 1993; Shin and Park, 1995; Yamamoto *et al*., 2000; Hirakawa *et al*., 2003; Goh *et al*., 2004; Jubelin *et al*., 2005). These findings have prompted many laboratories to examine the role of the EnvZ/OmpR system in the physiology of pathogenic bacteria. This TCS has been identified in a number of pathogens, including pathogenic *E. coli*, and bacteria of the genera *Shigella* and *Salmonella*, where it participates in the regulation of target genes in response to changes in osmolarity and pH, and is also involved in virulence (Bernardini *et al*., 1990; Bang *et al*., 2000; 2002; Lee *et al*., 2000; Schwan *et al*., 2002; Rentschler *et al*., 2013; Chakraborty *et al*., 2015). Microarray studies have revealed that OmpR influences the expression of as many as 125 genes in *E. coli* (Oshima *et al*., 2002) and 208 genes in *S. enterica* serovar Typhi (Perkins *et al*., 2013). Moreover, it has been shown that although the OmpR proteins of *E. coli* and *S. enterica* serovar Typhimurium are identical, the OmpR regulons in these enteric bacteria are divergent, with only 15 genes in common (Quinn *et al*., 2014).

The EnvZ/OmpR system also operates in the bacteria of the genus *Yersinia*, where it may serve a variety of functions. Some members of the *Yersinia* OmpR regulon have been identified by comparing gene expression in null *ompR* mutants with that in wild-type strains. The physiological consequences of the loss of the OmpR protein in *Y. enterocolitica* were studied by monitoring the growth and survival of cells subjected to various environmental stresses (Dorrell *et al*., 1998; Brzostek *et al*., 2003). These experiments provided evidence that OmpR is involved in the adaptation of *Y. enterocolitica* to high osmolarity, oxidative stress and low pH. OmpR was also found to be required for adaptation to osmotic upshifts and low pH in *Y. pseudotuberculosis* (Flamez *et al*., 2008; Zhang *et al*., 2013) and *Y. pestis* (Gao *et al*., 2011). These data confirmed that apart from its well-known role in the molecular response to changes in osmolarity, OmpR influences the expression of other environmental stress response genes, especially those encoding acid-induced proteins. OmpR of *Y. pseudotuberculosis* has been shown to positively regulate urease production, conferring the ability to resist acid stress conditions (Hu *et al*., 2009a). Studies on the role of OmpR in *Y. pseudotuberculosis* uncovered its function in the regulation of a Type VI secretion system that promotes resistance to low pH (Gueguen *et al*., 2013). OmpR is also involved in the positive regulation of flagella synthesis in *Y. enterocolitica* and *Y. pseudotuberculosis*, which contrasts with its negative role in *E. coli* (Hu *et al*., 2009b; Raczkowska *et al*., 2011a). Our laboratory has previously shown that OmpR inhibits transcription of the invasin gene *inv* in *Y. enterocolitica* (Brzostek *et al*., 2007). In a recent study we also observed a correlation between serum resistance of *Y. enterocolitica* and the activity of OmpR, suggesting that OmpR-dependent changes in outer membrane proteins (OMPs) and surface-anchored components might be responsible for this phenomenon, which could assist this bacterium in switching between distinct niches within and outside the host body (Skorek *et al*., 2013).

Environmental factors like temperature, and calcium and ferric ion concentrations, have a considerable impact on the production of membrane proteins, including virulence factors, in pathogenic Yersiniae (Straley and Perry, 1995). Proteomic studies on *Y. pestis* have examined

changes in membrane or soluble proteins in response to temperature and calcium (Chromy *et al*., 2005; Pieper *et al*., 2009a,b). However, the effect of environmental signals and the influence of the EnvZ/OmpR pathway, OmpR alone or other TCSs on the membrane proteome composition has yet to be studied in *Yersinia* spp.

In this study, alterations in the OMPs profile of *Y. enterocolitica* in response to the level of OmpR and varying temperature, pH or osmolarity were examined by comparative proteomic analysis. The shotgun proteomic analysis method was applied to permit quantification of any observed differences in the membrane proteome. Principal component analysis (PCA) of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) expression list was used to evaluate distinct abundance patterns among the analysed groups. Our results indicate that the loss of OmpR affects the production of 120 proteins, both positively and negatively. The impact of OmpR on the expression of the adhesin YadA and the HemR heme uptake receptor – identified as new OmpR-regulated targets – was studied in more detail.

Results and discussion

Proteomic analysis of outer membrane-enriched sarkosyl-insoluble membrane fractions (OMsl) of Y. enterocolitica strains

To investigate the role of the response regulator OmpR in modulating the OM composition of *Y. enterocolitica*, we performed a proteomic analysis of OMsl of wild-type strain Ye9 and isogenic *ompR* null mutant AR4, cultured at 26°C or 37°C in standard Luria–Bertani (LB) medium (86 mM NaCl, pH 7.0), or in LB supplemented with NaCl (386 mM, pH 7.0) or adjusted to pH 5.0. Shotgun label-free quantitative LC-MS/MS analysis of all OMsl samples produced a dataset of 543 proteins identified by at least two peptides. Among these proteins, the majority are annotated in the databases as cell envelope proteins, i.e. inner membrane (IM; 52%) and integral OM and OM-associated proteins (20%). The membrane proteins account for approximately 67% (OM) and 38% (IM) of the predicted respective membrane proteomes of *Y. enterocolitica*. We also identified periplasmic and cytoplasmic proteins, and proteins of unknown localization within the samples. Contamination by proteins localized outside the cell envelope is unavoidable because the lysis of bacterial cells leads to aggregation of the cellular contents. The presented data confirm the enrichment of OM proteins in the samples and support the validity of the procedure applied to isolate the OMsl fractions.

The proteomes of strains grown under different conditions were further compared to produce differential OMsl proteome lists (Tables S3 and S4).

Effect of temperature, osmolarity and pH on the membrane proteome of the wild-type Y. enterocolitica strain Ye9

As a first step in our differential analysis of the OMsl proteome of *Y. enterocolitica*, samples from wild-type strain Ye9 grown under different osmolarity and pH conditions at 26°C or 37°C were qualitatively and quantitatively compared (Table S3, Appendix S1). Proteomic analysis revealed 76 differentially expressed proteins accepted for quantification (q-value ≤ 0.05, at least two peptides per protein) following growth of *Y. enterocolitica* under the different conditions.

The greatest impact on the OM proteome was observed in response to pH (44 proteins whose abundance changed at pH 5.0) followed by temperature (39 changes) and osmolarity (26 changes), with several proteins affected by more than one physicochemical condition.

Temperature affected several proteins, particularly those involved in virulence. For example, the major *Y. enterocolitica* adhesin YadA (Skurnik and Toivanen, 1992) and components of the *Yersinia* Ysc-Yop T3SS (Lambert de Rouvroit *et al*., 1992; Akopyan *et al*., 2011) were more abundant at the higher temperature, in agreement with previous reports. The group of osmoregulated proteins included porin OmpC, in agreement with previous reports for *Y. enterocolitica* (Brzostek *et al*., 1989) and also *E. coli* (Russo and Silhavy, 1990). The proteins upregulated by low pH included the urease components UreA and UreG and the OM usher protein MyfC involved in *Y. enterocolitica* Myf fimbrial assembly, confirming previous reports (Iriarte and Cornelis, 1995; Hu *et al*., 2009a). In summary, this analysis detected several changes in protein abundance known to occur in response to different growth conditions, which confirmed the ability of this method to identify temperature, osmo- and acid-regulated cellular components. Together, the changes detected in the OMsl proteome of *Y. enterocolitica* may reflect physiological adaptations necessary for growth of Yersiniae in highly variable environments.

Differences in protein abundance between ompR mutant and parental strains

We next focused our analysis on proteins within the OMsl fraction that showed significant differences in abundance in the *ompR* mutant AR4 compared with the wild-type Ye9, cultured at 26°C or 37°C under different osmolarity and pH conditions. Statistical analysis of the quantitative results of the MS analysis revealed 120 proteins (q-value ≤ 0.05, identified by at least two peptides) showing differential abundance in the *ompR* mutant compared with the wild-type strain under at least one of the tested conditions (ratio ≤ 0.67 or ≥ 1.5, Table S4). Notably, differences between these two strains in the abundance of particular

proteins were observed upon growth in all three media, i.e. standard conditions, high osmolarity and low pH (36 proteins), while for other proteins, differences were evident only at high osmolarity and/or low pH (Fig. S1). Similarly, some differences were seen only at 26°C or 37°C (Fig. S1). To assess the variation in protein abundance patterns in both strains under the tested growth conditions (confirmed by independent biological repetition), we performed the multivariate statistical test called PCA (Fig. S2; Friedman *et al*., 2006; Friedman *et al*., 2007). First, two principal components indicated that temperature was a larger source of variation within the dataset than the *ompR* mutation (Fig. S2A). The different relative orientations of the group analyses at 26°C and 37°C under standard conditions (Fig. S2B), high osmolarity (Fig. S2C) or low pH (Fig. S2D) demonstrated high reproducibility between replicate samples and most likely indicated that a different subset of proteins was expressed. The PCAs confirmed the major differences between the protein expression patterns of the wild-type Ye9 and mutant AR4 at both temperatures under the different conditions of osmolarity and pH.

Of the 120 OmpR-dependent *Y. enterocolitica* proteins identified by proteomic analysis (Table 1, Table S4), the majority are annotated in the Swiss-Prot database as integral OM and OM-associated proteins (38%). Proteins from the IM (37%), periplasm (7%), cytoplasm (12%) and those of unknown localization (6%) were also identified. Some of the OmpR-dependent proteins recognized in this study are specific to the pathogenic Yersiniae (Ysc-Yop T3SS), others are present in different enteropathogenic Yersiniae, i.e. *Y. pseudotuberculosis* and *Y. enterocolitica* (Inv, YadA), while a few are found only in *Y. enterocolitica* serotype O:9 (O-Antigen biosynthesis enzymes WbcV, WbcU, WbcT).

The proteins regulated by OmpR (positively or negatively) were grouped into several Gene Ontology (GO) categories according to the biological processes in which they participate (Fig. 1, Table 1). About one third of the OmpR-dependent proteins are involved in transport across membranes, i.e. transporter activity (26%) and porin activity (7%). The next most abundant category corresponds to proteins involved in pathogenesis (17%), followed by proteins participating in cell envelope organization (12%) [including outer membrane assembly (5%), cell wall organization (4%) and LPS-associated O-antigen biosynthesis (3%)]. Proteins facilitating iron ion homeostasis were also identified (5%), as well as some involved in resistance to stress (5%). Thus, many OmpR-dependent proteins appear to play a role in the interaction of *Y. enterocolitica* with its surroundings.

The relatively large number of differentially expressed proteins identified by this proteomic analysis supports the previously suggested involvement of OmpR in global gene regulation in enterobacteria (Oshima *et al*., 2002; Perkins *et al*., 2013; Quinn *et al*., 2014). The assembled panel of proteins is likely to include some whose differential abundance results from direct regulation by OmpR, i.e. binding of this factor to promoter regions of the corresponding genes, while the expression of others might be affected indirectly through the influence of OmpR on other transcriptional regulators, post-transcriptional regulators (e.g. small RNAs) or even proteases. OmpR regulates multiple genes in the *Enterobacteriaceae* by binding to sites in their promoter regions that have similar but not identical sequences (Maeda *et al*., 1991; Harlocker *et al*., 1995; Huang and Igo, 1996; Yoshida *et al*., 2006; Rhee *et al*., 2008; Perkins *et al*., 2013). Binding site degeneracy makes the identification of new OmpR regulon members difficult. Nevertheless, we used the *E. coli* OmpR consensus sequence [TTTTA**C**TTTTT**G**(A/T)AA**C**ATAT] (Fig. 2A) (Maeda *et al*., 1991) to search for candidate genes of *Y. enterocolitica* regulated by OmpR among those encoding proteins identified by our proteomic analysis. Moreover, we also compared these promoters with a *Yersinia* consensus motif that was defined using sequences experimentally shown to bind OmpR (Fig. 2B). The predicted OmpR-DNA binding sites in the promoters of the indicated *Y. enterocolitica* genes (with highest similarity to the *E. coli* and *Yersinia* spp. consensus sequences) are listed in Fig. 2C. The fold change in the abundance of the identified OmpR-dependent proteins is shown graphically in Fig. 2D. Below, we describe the experimental testing of two of the putative elements identified by this *in silico* analysis using an *in vitro* DNA binding assay. In future it will be necessary to verify that the other genes with putative OmpR binding sequences are indeed the object of direct transcriptional control by this regulator. In the following sections we give a more detailed description of some of the identified OmpR-dependent proteins and provide some insights into the impact of this regulator on the adaptive abilities of *Y. enterocolitica*.

OmpR influences the production of general and substrate-specific porins

Eight proteins affected by OmpR were classified as porins (Table 1). They are homologues of general (i.e. non-specific) and substrate-specific porins from *E. coli* that form water-filled channels which permit the diffusion of hydrophilic solutes across the outer membrane (Nikaido, 2003). These proteins include the general porins OmpC and OmpF involved in the passive diffusion of small molecules (< 600 Da). We previously showed that both porins form hydrophilic diffusion channels across the OM of *Y. enterocolitica*, and that their absence reduces the permeability of the OM for β-lactam compounds (Brzostek and Nichols, 1990). In the present analysis, these two proteins were found to be less abundant in the *ompR*

Table 1. OMsl proteins differentially expressed in wild-type *Y. enterocolitica* strain Ye9 and isogenic *ompR* mutant AR4 cultured under different growth conditions.

Differentially expressed proteins		Regulation Ye9 versus AR4[b]					
		Standard conditions		High osmolarity		Low pH	
Accession no.	Protein description[a]	26°C	37°C	26°C	37°C	26°C	37°C
Porin activity GO:0015288							
ADZ43059	Putative outer membrane porin protein F, OmpF	11.6		8.03		8.16	3.3
ADZ43215	Outer membrane porin protein C, OmpC		3.86	7.39	1.97	10.2	2.98
ADZ42354	Outer membrane phosphoporin protein E, PhoE	7.28	2.08	7.07		5.64	
ADZ41941	Outer membrane protein X, OmpX					5.69	
ADZ44282	Oligogalacturonate-specific porin KdgM2	–120.03	–10.57	–183.86	–4.1	–145.25	–15.68
ADZ42758	Outer membrane protein W, OmpW				–2.31		–3.72
ADZ40635	Vitamin B12 transporter BtuB				–2.03		
ADZ41063	Sucrose porin ScrY	2.76	–8.44				–5.33
Transporter activity GO:0005215							
ADZ42555	Dipeptide and tripeptide permease A, DtpA/TppB	15.81				16.4	
CBY28945	Anaerobic C4-dicarboxylate transporter DcuA	10.89		16.39		14.67	
ADZ44176	Nitrite transporter NirC				6.05		
ADZ42774	Periplasmic oligopeptide-binding protein OppA	5.36		2.8		4.27	
ADZ42770	Oligopeptide transport ATP-binding protein OppF			2.98			
ADZ42771	Oligopeptide transporter ATP-binding component OppD			2.53			
ADZ44050	Putative xanthine/uracil permease			2.13		2.32	
ADZ41508	Outer membrane efflux protein	2.78		1.8			
ADZ41657	Multidrug efflux protein AcrA				1.49*		2.13
ADZ41656	Multidrug efflux protein AcrB		1.24*				
ADZ43362	ABC transport system substrate-binding protein	2.91		3.46		2.71	
ADZ44078	Maltose ABC transporter periplasmic protein MalE	2.54				2.17	
ADZ44153	Putative sugar transferase		2.65				
ADZ42972	Glucose-specific PTS system IIBC components			1.85		1.83	
ADZ41295	Protein translocase subunit SecA		1.66		1.78		
ADZ42257	D-alanine/D-serine/glycine permease CycA	–37.02	–32.2	–33.54		–44.45	
ADZ41741	Putative glutamate/aspartate transport system permease			–5.12		–12.54	
ADZ42241	Proline permease		–11.31				
ADZ43898	Serine/threonine transporter SstT	–10.49		–10.88			
ADZ40803	Cation/acetate symporter ActP	–7.12		–9.75		–7.57	
EHB19555	Amino acid permease					–9.39	
ADZ41742	Glutamate and aspartate transporter subunit	–4.21		–7.46		–5.57	
ADZ41044	Arginine/ornithine antiporter	–2.48				–2.21	
ADZ42170	Mg(2+) transport ATPase protein B	–8.3	–4.25	–9.64		–7.55	
ADZ43328	Long-chain fatty acid outer membrane transporter FadL		–4.71	–3.89	–7.46	–4.69	–5.66
EOR82078	Putative phosphotransferase system protein		–5.04		–3.74		–4.61
ADZ44370	PTS system, mannitol-specific IIABC component						–1.81
ADZ43484	PTS system, glucitol/sorbitol-specific IIBC component	–4.2	–3.64				
ADZ41346	Chloride channel protein ClcA	–2.23	–4.18			–2.06	
ADZ43615	Voltage-gated potassium channel	–2.36					
ADZ41961	D-galactose-binding periplasmic protein MglB		–3.37				
ADZ41046	RND family efflux transporter			–1.88			
ADZ43857	Type I secretion outer membrane protein TolC	–1.39*	–1.65		–1.61	–1.62	–1.57
ADZ41495	DL-methionine transporter substrate-binding subunit		2.38	–2	2.86		2.9
Gram-negative-bacterium-type cell outer membrane assembly GO:0043165							
ADZ43450	Outer membrane protein assembly factor BamC	2.72	1.78	2.07		2.44	1.8
ADZ41154	Outer membrane protein assembly factor BamD					2.01	
ADZ41474	Outer membrane protein assembly factor BamA	1.38*		1.35*		1.35*	
ADZ42323	Outer membrane lipoprotein LolB						–4.5
ADZ41736	LPS-assembly lipoprotein LptE				–1.74		–1.5
ADZ41135	LPS-assembly protein LptD		–1.54	1.37*	–1.79		–1.43*
Biosynthetic process GO:0009058							
ADZ42004	WbcT protein	1.73		2.29	2.74	2.24	2.91
ADZ42006	WbcV protein		2.57				
ADZ42005	WbcU protein			1.93		1.95	
Cell wall organization GO:0071555							
ADZ42523	Murein L,D-transpeptidase		2.49				
ADZ42453	N-acetylmuramoyl-L-alanine amidase	1.82	2.13				1.81
EOR80052	Major outer membrane lipoprotein Lpp	1.79		1.47*	1.98	1.9	2.05
ADZ41339	Penicillin-binding protein 1b						1.93
ADZ41447	Membrane-bound lytic murein transglycosylase A			–2.07			
Pathogenesis GO:0009405							
ADZ43157	Outer membrane usher protein MyfC						6.24
ADZ42189	Invasin, Inv	1.87	1.71	1.49*			

Table 1. *cont.*

Differentially expressed proteins		Regulation Ye9 versus AR4[b]					
		Standard conditions		High osmolarity		Low pH	
Accession no.	Protein description[a]	26°C	37°C	26°C	37°C	26°C	37°C
ADZ44444	Transmembrane effector protein YopB		–48.12		–42.4		–75.73
ADZ44443	Translocator protein YopD		–40.37		–37.76		–71.6
ADZ44440	Type III secretion system effector protein YopM		–20.79		–42.45		
ADZ44516	Protein kinase YopO		–16.25		–9.64		–22.77
ADZ44518	Type III secretion system effector protein YopP		–10.37		–11.94		–19.12
EOR65641	Type III secretion system effector protein YopE		–5.77		–8.41	–3.96	–15.03
ADZ44479	Tyrosine-protein phosphatase effector protein YopH		–6.78		–8.49		–13.32
ADZ44435	Type III secretion system effector protein YopT		–4.12				
ADZ44434	Type III secretion modulator of injection YopK/YopQ		–18.54		–21.11		–33.89
ADZ44454	Type III secretion outer membrane protein YopN		–14.62		–14.4		–17.46
ADZ44467	Type III secretion OM pore forming protein YscC	–6.63	–7.48	–16.2	–6.37	–10.1	–7.66
ADZ44451	Type III secretion protein YscX		–11.89				
ADZ44455	Type III secretion apparatus H+-transporting two-sector ATPase YscN		–2.87				–3.23
ADZ44457	Type III secretion system needle length determinant YscP		–2.78				
ADZ44497	Adhesin YadA	–4.97	–9.83	–10.37	–10.81	–5.02	–10.27
ADZ40701	Phospholipase A, YplA		–2.07		–2.37		–2.06
ADZ43625	Urease subunit gamma UreA			–2.44			2.2
ADZ43623	Urease subunit alpha UreC		2.43	–2.03	2.89	–1.28*	5.4
ADZ43620	Urease accessory protein UreG	–2.5	1.99		2.05	–1.86	2.65
Iron ion homeostasis GO:0055072							
ADZ43721	Ferric anguibactin-binding protein FatB				2.31		
ADZ41314	Outer membrane receptor FepA	1.68					
ADZ41093	Heme ABC exporter, ATP-binding protein CcmA		1.59				
ADZ41067	Iron transporter FecA	–2.08	–2.56	–2.53		–2.3	–2.96
ADZ40857	Hemin receptor HemR		–2.02				–1.72
ADZ44135	Bacterioferritin Bfr		10.28		8.15	–4.57	7.17
Response to stress GO:0006950							
ADZ42566	Phage shock protein PspA						2.36
ADZ41933	DNA protection during starvation protein			2.23			
ADZ41491	Copper homeostasis protein CutF			–2.03			–2.16
ADZ42722	Putative carbon starvation protein A, CstA		–2.99	–1.94	–2.43	–1.58	–3.24
ADZ41113	Chaperone protein DnaK, Hsp70	–1.56	–2.01				–1.64
ADZ42757	Osmotically inducible protein Y			–2.27			
ADZ43049	Paraquat-inducible protein B						1.9
Catalytic activity GO:0003824							
ADZ43177	Inner membrane protein YeiU	12.47					
ADZ41168	Signal recognition particle protein			1.83			
ADZ40865	Keto-acid formate acetyltransferase				1.61		
ADZ43088	Formate acetyltransferase 1		1.51				
ADZ42412	Long-chain-fatty-acid-CoA ligase FadD	–11.73	–4.32			–5.15	–4.44
ADZ40899	Protein HflC	–3.28					
ADZ42794	Protease 4		–1.67				
Cell motility GO:0048870							
ADZ42196	Flagellar hook protein FlgE						3.07
ADZ42168	Putative methyl-accepting chemotaxis protein	–5.05					
ADZ42180	Methyl-accepting chemotaxis protein	–2.32					
ADZ42216	Flagellar M-ring protein					–1.71	
Cell redox homeostasis GO:0045454							
ADZ41797	Cytochrome D ubiquinol oxidase subunit II					2.56	
ADZ41796	Cytochrome D ubiquinol oxidase subunit I	1.48*		1.83		2.33	
ADZ42602	NAD(P) transhydrogenase subunit alpha			1.77			
ADZ41617	Cytochrome O ubiquinol oxidase subunit II			–1.52		–1.4*	–1.51
Cell division GO:0051301							
ADZ43396	Cell division protein ZipA homologue	–3.52		–2.15			
ADZ41291	Cell division protein FtsZ	–1.9					
Undefined GO term							
ADZ41569	Putative exported protein						14.98
ADZ40718	Putative membrane protein	7.34		5.61		7.44	
ADZ43361	Putative exported protein					2.53	
ADZ43548	Putative lipoprotein YfhG						2.47
ADZ44035	Outer membrane lipoprotein PcP						1.86
ADZ43116	Putative lipoprotein					1.51	
ADZ40804	Inner membrane protein YjcH	–9.68	–35	–10.62		–12.42	–37.22
ADZ41451	Lipoprotein			–4.43			

Table 1. *cont.*

Differentially expressed proteins		Regulation Ye9 versus AR4[b]					
		Standard conditions		High osmolarity		Low pH	
Accession no.	Protein description[a]	26°C	37°C	26°C	37°C	26°C	37°C
ADZ43231	Outer membrane protein YfaZ				–4.17		–3.89
ADZ41832	Uncharacterized protein					–3.97	
ADZ41163	Putative exported protein			–3.28		–2.61	
ADZ42504	Lipoprotein NlpC			–3.22			
ADZ41640	Lipoprotein, YscW superfamily			–2.43		–1.87	
ADZ43738	Putative outer membrane lipoprotein			–2.1			
ADZ42938	Putative exported protein	1.75				2.12	–2.16

a. Description of the identified proteins of OMsl (outer membrane-enriched sarkosyl-insoluble fractions) according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Proteins were clustered based on Gene Ontology (biological process) terms.
b. Proteins whose abundance differed between the wild-type strain Ye9 and OmpR-deficient mutant AR4, according to MS analysis. Within each category, the proteins are sorted according the effect of OmpR: positive followed by negative (ranked from highest to lowest fold change). Standard conditions (LB medium); high osmolarity (LB supplemented with NaCl to 386 mM); low pH (LB adjusted to pH 5.0), at 26°C and 37°C; *q*-value ≤ 0.05; '–' protein more abundant in *ompR* mutant strain, fold change is shown; *value of fold change slightly different from the accepted threshold value of 1.5.

mutant compared with the wild-type (Table 1), which confirms our previous finding (Brzostek and Raczkowska, 2007) and supports the notion that OmpR is required for the activation of these genes in both *E. coli* and *Y. pestis* (Russo and Silhavy, 1990; Gao *et al.*, 2011). The levels of OmpC and OmpF in the OM of enterobacteria vary depending on the osmolarity of the medium. In the *E. coli* model, the osmoregulation of both porins is mediated by EnvZ/OmpR so that OmpC (the narrow porin) levels increase in media of high osmolarity, while those of OmpF (the wider porin) decrease (Forst and Inouye, 1988). It is thought that this alteration in membrane protein composition may limit the diffusion of harmful compounds into cells growing within a mammalian host. The observed osmoregulation has been correlated with the strength of OmpR binding to three and four consensus-like sequences identified in the DNA regions upstream of the *E. coli ompC* and *ompF* open reading frames (ORFs) respectively. In the wild-type *Y. enterocolitica* strain Ye9, we found an increased level of OmpC at high osmolarity, while OmpF abundance was not affected (Table S3). Notably, three consensus-like *OmpR*-binding site sequences were identified within the regulatory regions of the *Y. enterocolitica ompC* and *ompF* genes (Fig. 2), as was also the case in pathogenic *Yersinia* (Gao *et al.*, 2011). Thus, the lack of osmoregulation of the *ompF* gene in *Y. enterocolitica* Ye9 might be correlated with the absence of a distal fourth OmpR-binding site in the promoter. The pattern of porin osmoregulation in *Y. enterocolitica* is clearly different from that of *E. coli*, but is shared by *S. Typhi and Y. pestis* (Puente *et al.*, 1991; Gao *et al.*, 2011). Together, these results indicate that some features of the regulation of *ompC* and *ompF* expression, such as dependence on OmpR, appear to be common to these bacteria, although the osmoregulatory mechanism seems to be different. Such variations in porin regulation among different enterobacteria might reflect the varied function of these proteins in bacteria growing in different environmental niches. Since the OmpC porin seems to play some role in the adhesion properties of *Y. enterocolitica* (Raczkowska *et al.*, 2011b), the increased level of this protein at high osmolarity could be beneficial to cells residing in the ileum.

The third general porin upregulated by OmpR is similar to the anion-specific phosphoporin PhoE induced by phosphate deprivation in *E. coli* (Nikaido, 2003). To our knowledge, a link between PhoE and OmpR has not previously been identified in *E. coli* and thus might reflect a specific adaptation of *Y. enterocolitica* physiology, especially at low ambient temperature. However, we were unable to identify a consensus OmpR-binding site in the *phoE* promoter. OmpR also influenced the production of OmpX, a porin of undefined function, in agreement with previous data demonstrating the positive regulation of *ompX* expression by OmpR in *Y. enterocolitica* (Skorek *et al.*, 2013) and *Y. pestis* (Gao *et al.*, 2011). Inspection of the regulatory region of *ompX* showed two putative OmpR binding sites with 45% and 60% identity to the *E. coli* consensus sequence, and 45% and 50% identity to the *Yersinia* spp. consensus sequence respectively (Fig. 2). The panel of OmpR-dependent porins also included a sucrose-specific porin related to enterobacterial ScrY (Schmid *et al.*, 1991) and OmpW, a small porin of the OmpW/AlkL family present in all Gram-negative bacteria, which might be involved in the response to different stresses, e.g. osmotic and oxidative stress (Hong *et al.*,

Fig. 1. Functional characterization of *Y. enterocolitica* proteins whose abundance is regulated by OmpR (see online version for colours).
A. Classification of proteins differentially expressed in the wild-type strain Ye9 compared with OmpR-deficient mutant AR4 under all tested conditions, using Gene Ontology (GO) biological process. The classification is based on BioCyc Database Collections, the UniProt databases and literature data.
B. Classification of differentially expressed proteins associated with biological transport processes.
C. Chart indicating the number of differentially expressed proteins that are upregulated (more abundant) or downregulated (less abundant) in the *ompR* mutant AR4 compared with the wild-type Ye9, divided according to biological process classification.
D. Chart indicating the number of differentially expressed proteins associated with biological transport processes that are upregulated or downregulated in *ompR* mutant AR4 compared with the wild-type Ye9.

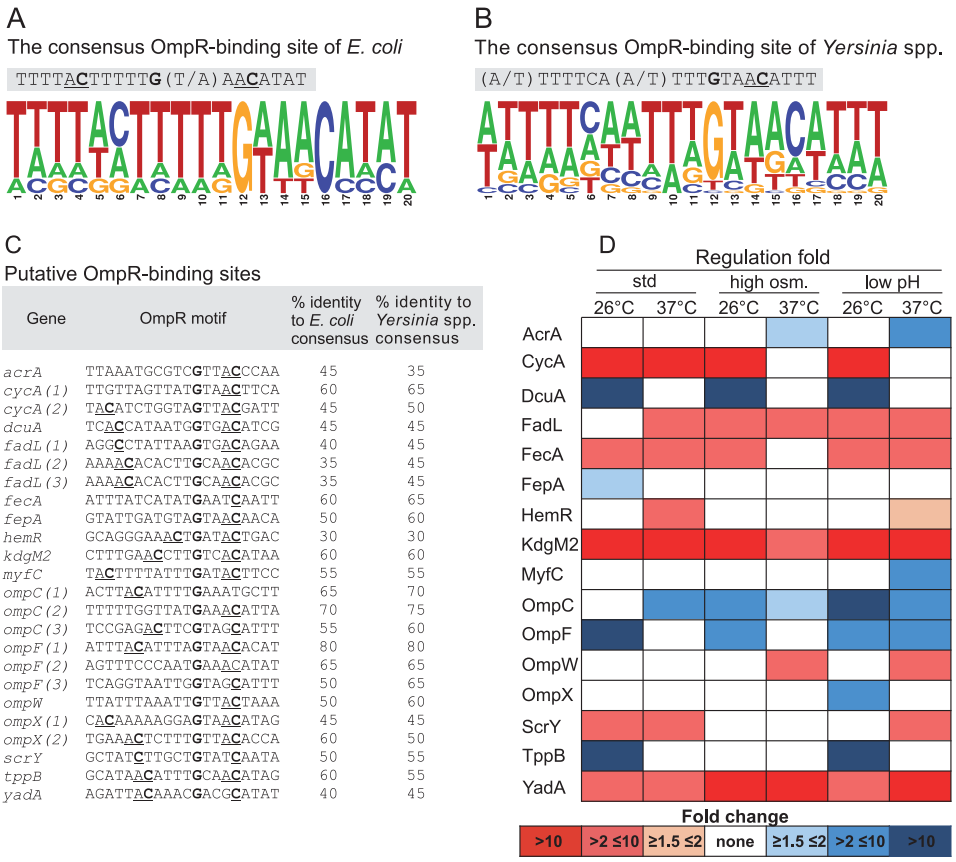


Fig. 2. Putative OmpR-binding sites identified in the promoter regions of *Y. enterocolitica* genes encoding OmpR-dependent proteins (see online version for colours).
A. The consensus OmpR-binding site of *E. coli* and logo motif defined based on analysis of OmpR-binding elements in the *ompC* and *ompF* promoter regions (Maeda *et al.*, 1991).
B. The consensus OmpR-binding site of *Yersinia* spp. and logo motif defined based on experimentally validated OmpR-binding elements present in the promoter regions of *inv* (Brzostek *et al.*, 2007), *flhDC* (Hu *et al.*, 2009b; Raczkowska *et al.*, 2011a), *acrR* and *acrAB* (Raczkowska *et al.*, 2015), and *ompC*, *ompF*, *ompR* and *ompX* (Gao *et al.*, 2011). WEBLOGO (http://weblogo.berkeley.edu/logo.cgi) was used to obtain consensus sequence logos in which the height of individual letters within the stack of letters represents the relative frequency of that particular nucleotide at a given position, and the number of letters in the stack indicates the degree of conservation at that position.
C. Sequences of putative OmpR-binding sites in the promoters of the indicated *Y. enterocolitica* genes determined based on similarity to the consensus sequences of *E. coli* and *Yersinia* spp. (percentage identity values are shown). The central motif **G**XX**AC** or **G**XXX**C** and the **AC** or **C** nucleotides usually located about 10 nt away from the **AC** elements of the central motif are marked.
D. Graphical representation of the fold change in the abundance of the identified OmpR-dependent proteins under the tested growth conditions. Proteins that are more or less abundant in the *ompR* mutant strain are indicated by the scale, which shows pale to dark colours of red and blue respectively.

2006). Putative OmpR-binding sites were identified in the promoter regions of the genes encoding these proteins (Fig. 2).

A major impact of OmpR on the proteomic profile of *Y. enterocolitica* was its effect on the abundance of porin KdgM2 related to KdgM and KdgN oligogalacturonate-specific porins in *Dickeya dadantii* (Blot *et al.*, 2002, Rodionov *et al.*, 2004). Upon growth at 26°C, the OmpR-negative strain exhibited a more than 100-fold increase in the level of this protein (Table 1), implying a major role for OmpR in repressing KdgM2 production. A putative OmpR-binding motif was recognized in the promoter region of *kdgM2* (Fig. 2). In the pectinolytic bacterium *D. dadantii* KdgM and KdgN porins overlap functionally, and their expressions are subject to reciprocal OmpR regulation, although the direct involvement of OmpR in this process was not verified (Condemine and Ghazi, 2007).

Transporters affected by OmpR

As stated above, the majority (24%) of proteins identified as OmpR-regulated belong to the GO category of proteins with 'Transporter activity' (Fig. 1, Table 1). Strikingly, OmpR appears to exert a negative influence on amino acid uptake (seven proteins upregulated in the *ompR* mutant compared with the wild-type) while promoting peptide uptake (downregulation of TppB, OppA, OppD and OppF in the *ompR*-negative strain). The D-alanine/D-

serine/glycine permease CycA deserves a special mention since this protein exhibited a more than 30-fold increase in *ompR* mutant cells. Since D-alanine is a central molecule in peptidoglycan assembly and cross-linking (Walsh, 1989), the OmpR-dependent negative regulation of a D-alanine/D-serine/glycine permease might be relevant for the cell wall metabolism of *Y. enterocolitica*. Conversely, the tripeptide permease TppB was less abundant (~ 15-fold) in the *ompR* cells compared with the wild-type strain (Table 1), which is in agreement with data obtained in *S. Typhimurium* and *E. coli* showing that OmpR is involved in the positive regulation of *tppB* (Gibson *et al*., 1987; Goh *et al*., 2004). *In silico* analysis identified putative OmpR-binding sites in the promoters of both the *cycA* and *tppB* genes of *Y. enterocolitica* (Fig. 2).

We also found that the transport of exogenous long-chain fatty acids (LCFAs) across the *Y. enterocolitica* cell envelope could be modulated by OmpR, since the OM transporter FadL was four- to sevenfold more abundant in the *ompR* mutant than in the parental strain. This suggests that OmpR exerts a negative effect on FadL production, which is in agreement with a study that reported the inhibition of *fadL* transcription by OmpR in *E. coli* (Higashitani *et al*., 1993). Putative OmpR-binding sites were identified in the promoter region of the *Y. enterocolitica fadL* gene (Fig. 2). The OmpR-dependent modulation of LCFA uptake from the environment may be important for several cellular processes in *Y. enterocolitica*, including lipid metabolism.

Another transporter whose abundance was decreased (11- to 16-fold) in the *ompR* mutant is DcuA, an IM C_4-dicarboxylate transporter (antiporter for aspartate and fumarate) (Table 1). The *dcuA* and *dcuB* genes of *E. coli* encode homologous proteins that appear to function as independent C_4-dicarboxylate transporters under different growth conditions (Golby *et al*., 1998). The predicted OmpR-binding site in *Y. enterocolitica dcuA* is shown in Fig. 2.

Finally, the abundance of some efflux transporters was also altered in the *ompR* mutant (Table 1). For example, AcrA, a component of the AcrAB-TolC multidrug efflux pump, was less abundant in the *ompR*-negative strain AR4 compared with the parental strain Ye9. This efflux pump belongs to the RND family, some members of which confer drug resistance in Gram-negative bacteria (Blair and Piddock, 2009). A putative OmpR-binding site found in the *acrA* promoter region is shown in Fig. 2.

In summary, our results suggest that OmpR influences the expression of nutrient transporters to promote the uptake of peptides (while repressing amino acid uptake) and reduce the uptake of long-chain fatty acids. In addition, alterations in the membrane protein composition mediated by OmpR may promote the excretion of toxic compounds, thereby mitigating their harmful effects. Some of the differentially expressed proteins are encoded by genes that have not previously been considered members of the OmpR regulon, and further work is required to identify those that are directly regulated by OmpR.

Outer membrane assembly: OMPs and LPS

Our proteomic analysis characterized the impact of OmpR on proteins belonging to the GO category 'Gram-negative-bacterium-type cell outer membrane assembly'. Importantly, three proteins of the Bam complex, i.e. BamA, BamC and BamD, were less abundant in the *ompR* mutant AR4 compared with the wild-type strain Ye9 (Table 1). In *E. coli*, BamABCD is a multisubunit complex in the outer membrane that is responsible for folding and inserting OMPs in a beta-barrel conformation (Rigel and Silhavy, 2012). The observed OmpR-dependent regulation of Bam proteins in *Y. enterocolitica* indicates a role for OmpR in modulating the protein composition of the outer membrane. In addition, we observed a two- to threefold decrease in the proteins WbcV, WbcU and WbcT in the *ompR* mutant (Table 1). These proteins are involved in the synthesis of the unique serotype O:9 O-polysaccharide (OP) present in the LPS of *Y. enterocolitica* Ye9 (Skurnik *et al*., 2007). In a previous study we revealed that the loss of OmpR correlates with a reduced LPS/OP content in the OM of *Y. enterocolitica* serotype O:9 (Skorek *et al*., 2013). Thus, OmpR could modulate the LPS status of *Y. enterocolitica* through its influence on WbcV, WbcU and WbcT. Since we were unable to identify OmpR-binding sites in the regulatory regions of the *bam* and *wbc* genes, the role of OmpR in modulating the production of these proteins is probably indirect.

Pathogenesis

The second most abundant GO category of OmpR-dependent proteins is related to pathogenesis (Fig. 1, Table 1). Almost all proteins in this category were downregulated by OmpR, with the notable exception of the OM usher protein MyfC, involved in *Y. enterocolitica* Myf fimbrial assembly. OmpR promoted the production of MyfC upon growth at 37°C and acid pH (Table 1), in agreement with the environmental parameters known to induce Myf antigen synthesis (Iriarte and Cornelis, 1995). The predicted OmpR-binding site in the *myfC* promoter region is shown in Fig. 2. Other interesting exceptions were components of urease, whose abundance was modulated either positively or negatively depending on the temperature. Urease is a multisubunit metalloenzyme that is crucial for resistance to low pH and promotes the

survival of *Y. enterocolitica* in the presence of stomach acid (De Koning-Ward and Robins-Browne, 1995). Our results revealed the positive impact of OmpR on urease expression in *Y. enterocolitica* grown at 37°C and the opposite effect in cells cultured at 26°C, i.e. negative OmpR-dependent regulation of UreA, UreC and UreG at the lower temperature. Urease genes were previously shown to be directly and positively regulated by OmpR in *Y. pseudotuberculosis* at 37°C, but lower temperatures were not assessed (Hu *et al*., 2009a). It is noteworthy that direct binding of OmpR to the promoter regions of urease genes has been demonstrated in *Y. pseudotuberculosis* (Hu *et al*., 2009a), but we were unable to identify consensus OmpR-binding sites in the regulatory regions of the three *ure* transcriptional units of *Y. enterocolitica* (*ureABC*, *ureEF* and *ureGD*). We speculate that during infection of the host (at 37°C), especially via the gastrointestinal route where the bacteria encounter gastric acid, OmpR promotes the production of urease to facilitate survival in the stomach and persistence in environmental niches of low pH in the later stages of pathogenesis.

The majority of the identified proteins in the Pathogenesis GO category are part of the Ysc-Yop T3SS (Table 1) and are encoded by virulence plasmid pYV. These include structural components of the *Yersinia* injectisome, the regulatory elements, the secreted effectors and translocators (Dewoody *et al*., 2013). Increased levels of all these proteins were detected in the *ompR* mutant at 37°C in at least one of the tested growth media (~ 3- to 70-fold). The differences in the levels of Ysc-Yop proteins related to the presence of OmpR were confirmed by Western blot analysis (data not shown). The regulation of Ysc-Yop expression in pathogenic *Yersinia* is highly complex and tightly connected with the secretion process, which is triggered at a temperature of 37°C in calcium-deficient medium and modulated by T3SS regulatory proteins and certain host signals *in vivo* (Straley *et al*., 1993; Li *et al*., 2014). However, since the growth conditions employed for this proteomic analysis were not designed to optimize Yop secretion (the growth medium was not depleted of calcium), it is unclear whether the observed alterations in the abundance of these proteins resulted from (i) a direct effect of OmpR on *ysc-yop* gene expression, (ii) some indirect effect due to changes in the cell envelope, or (iii) disturbance of the secretion process leading to accumulation of secreted proteins in the envelope. Definition of the precise role of OmpR in the regulation of *ysc-yop* gene expression will be the subject of future investigations. Finally, our proteomic analysis revealed that the loss of the OmpR regulator caused a five- to tenfold increase in YadA, a pYV-encoded, multifunctional OM protein (Table 1). Since YadA represents a major adhesin and serum resistance factor of *Y. enterocolitica* (El Tahir and Skurnik, 2001; Mikula *et al*., 2013), we decided to investigate the mechanism of OmpR-mediated downregulation of *yadA* expression in *Y. enterocolitica*.

OmpR downregulates expression of the major adhesin gene yadA

The proteomic data showed an increased amount of YadA in the *ompR* mutant strain AR4 compared with the wild-type strain Ye9 under all tested growth conditions (Table 1). The abundance of YadA protein in the OM of *Y. enterocolitica* cells grown at 26°C and 37°C under different osmolarity and pH conditions was evaluated further by Western blotting using a YadA-specific antibody (Fig. 3A and B). It has been shown previously that YadA is a trimeric protein exhibiting heat stability, with only slight denaturation during heating in Laemmli buffer (Mack *et al*., 1994; Schutz *et al*., 2010). To assess any differences in the levels of the oligomeric and monomeric forms of YadA between the wild-type strain Ye9 and the *ompR* mutant strain AR4, the OMsl samples were untreated or treated with 8 M urea to disrupt protein trimers. As shown in Fig. 3A OMsl samples from strains grown at 37°C, boiled in Laemmli buffer and examined by Western blotting, gave a YadA band of approximately 200 kDa and several bands of intermediate size. Moreover, the sample prepared from the *ompR* mutant AR4 showed an increased amount of YadA oligomers compared with the samples from wild-type strain Ye9. Western blot analysis of the OMsl fractions demonstrated that the quantity of the monomeric form of YadA resulting from urea denaturation was higher in the *ompR* mutant than the wild-type strain (Fig. 3B). In both analyses (with and without urea) the differences in the level of YadA between the two strains were observed independently of the osmolarity and pH conditions. YadA production was negligible in both strains cultured at 26°C, confirming the temperature-inducible nature of YadA and suggesting that the observed thermoregulation of this protein is OmpR-independent.

To obtain further evidence that OmpR regulates YadA expression, experiments were performed using plasmid pFX-yadA, which carries the *yadA* promoter driving the expression of a translational fusion of the first 16 codons of *yadA* with the gene encoding GFP. Plasmid pFX-0 carrying the promoterless *gfp* gene was used as a negative control (Schmidtke *et al*., 2013). Both plasmids were introduced into the wild-type and mutant *Y. enterocolitica* strains, and following growth under different temperature, pH and osmolarity conditions, bacterial fluorescence was measured by flow cytometry (Fig. 4). The cells carrying the control plasmid pFX-0 gave a low fluorescence signal (data not shown), in contrast to those transformed with pFX-yadA, encoding the YadA'–'GFP fusion. Higher fluorescence was observed in the *ompR* mutant strain AR4 compared with the wild-type Ye9 in cells grown to stationary phase at 37°C and 27°C (Fig. 4). Interestingly, the

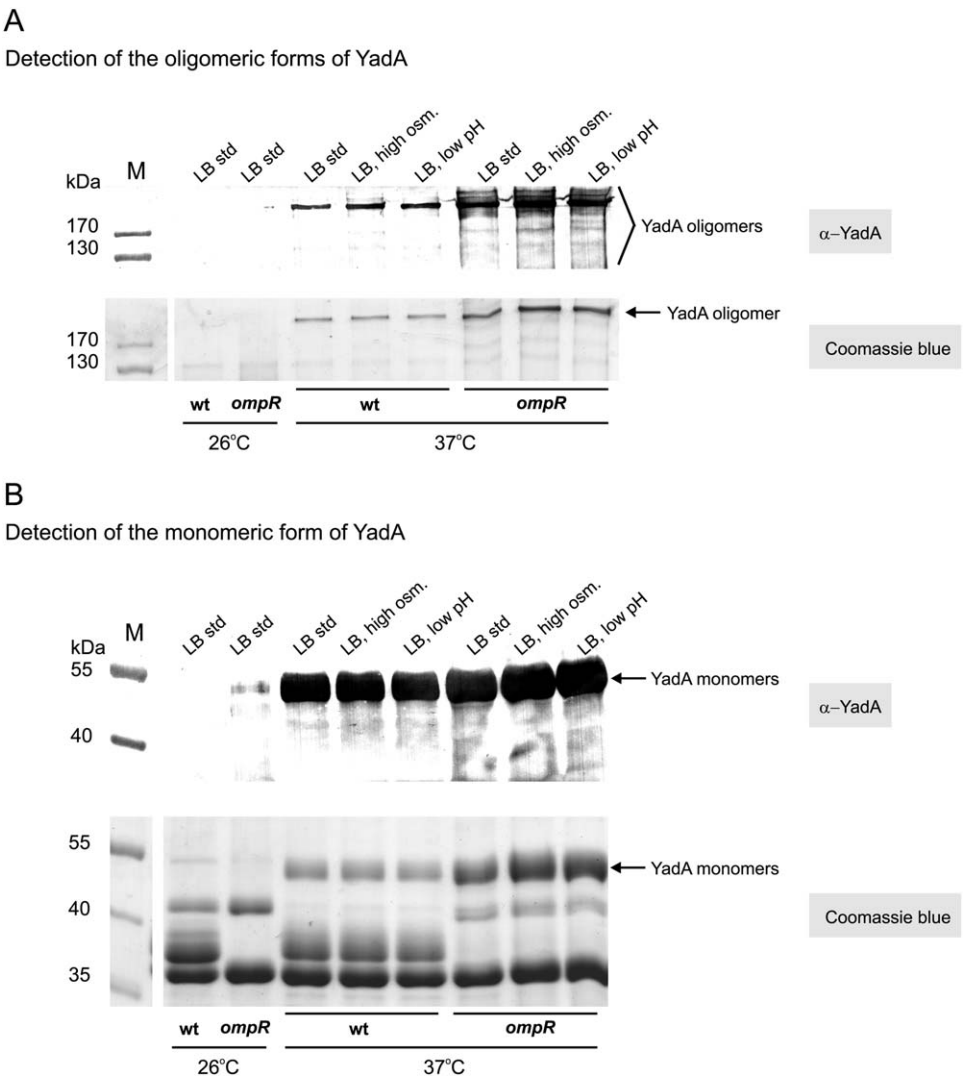


Fig. 3. OmpR-dependent YadA expression.
A. Immunodetection of the oligomeric forms of YadA protein in the outer membrane-enriched sarkosyl-resistant fractions of wild-type Ye9 (wt) and OmpR-deficient mutant AR4 (*ompR*) strains of *Y. enterocolitica*. The analysed samples were prepared from cells grown at 26°C in standard LB medium (std, 86 mM NaCl, pH 7.0), at 37°C in standard LB, in LB with raised osmolarity (high osm., 386 mM NaCl, pH 7.0) or in LB of low pH (low pH, 86 mM NaCl, pH 5.0). Samples were boiled for 5 min in Laemmli buffer before electrophoresis in a 10% polyacrylamide gel (SDS-PAGE). The top panel shows the immunoblot probed with a polyclonal antibody against YadA (α-YadA), and the bottom panel shows the Coomassie blue-stained gel as a loading control. On the Western blot, oligomeric YadA gives a band of approximately 200 kDa and several others of intermediate size. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This result is representative of least three independent experiments.
B. Immunodetection of the monomeric form of YadA protein. To disrupt protein trimers, samples were boiled with urea sample buffer prior to loading the gel. The top panel shows the immunoblot, and the bottom panel shows the Coomassie blue-stained gel. Wild-type strain Ye9 (wt) and OmpR-deficient mutant AR4 (*ompR*) were grown under the conditions described in (A). The band corresponding to YadA monomers on the Western blot (approximately 50 kDa) is shown. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This experiment was performed twice with similar results.

increase in YadA'–'GFP expression in the mutant strain was much greater than in the wild-type, especially at 37°C, in response to high osmolarity stress (threefold increase) (Fig. 4B). To confirm that the lack of OmpR resulted in derepression of *yadA*, plasmid pompR carrying the wild-type *ompR* allele was used to complement the mutation in strain AR4. Complementation caused reduced fluorescence in cultures grown in LB medium at both 27°C and 37°C (Fig. 4A and B), indicating that OmpR negatively regulates *yadA*. The complementation effect was not as clear in cells grown under high osmolarity and low pH.

A putative binding site for OmpR was identified 57 bp downstream of the transcription start of *yadA* by *in silico* analysis (Y1 site), suggesting that OmpR might directly repress *yadA* transcription (Figs 2 and 5A). To more precisely define the OmpR binding site, three fragments (F1,

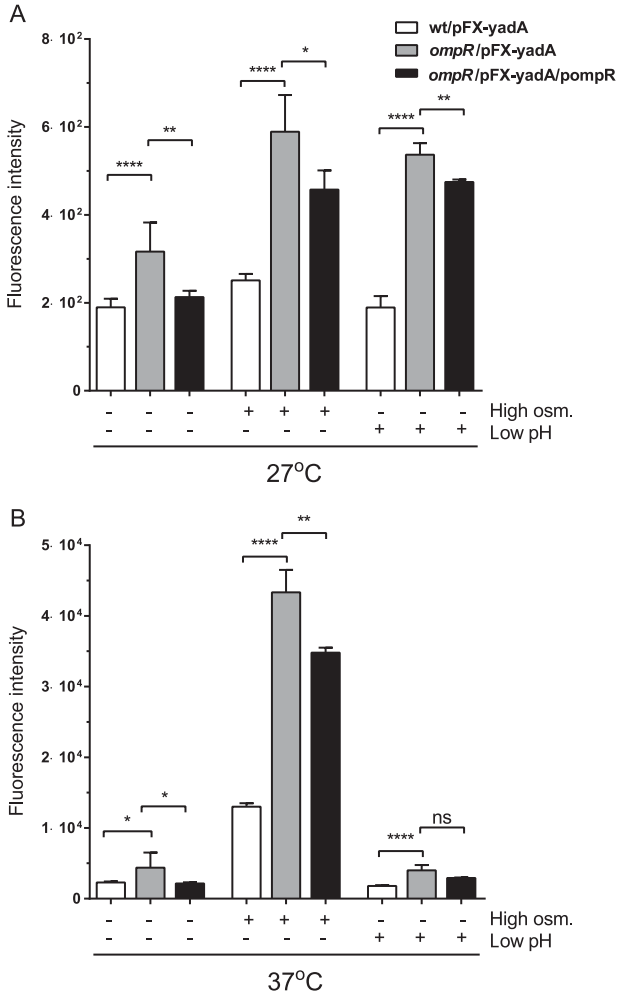


Fig. 4. Analysis of OmpR-dependent YadA expression using a YadA'–' GFP- translational fusion. Fluorescence intensity of Ye9 (wt), AR4 (*ompR*) and complemented strain AR4 (*ompR*/pompR) containing pFX-*yadA*, analysed by flow cytometry. All strains were grown to stationary phase in LB medium (standard conditions), LB supplemented with NaCl (386 mM NaCl, pH 7.0; high osm.) or LB adjusted to pH 5.0 (low pH), at 27°C (A) or 37°C (B). In these experiments, the mean fluorescence intensity of strains carrying a promoterless *gfp* gene (plasmid pFX-0) was between 8 and 14. The data represent mean values with the standard deviation from at least two independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using Student's unpaired *t*-test (***$P < 0.001$, *$P < 0.05$).

F2, F3) from the *Y. enterocolitica yadA* regulatory region were amplified by polymerase chain reaction (PCR) (Fig. 5B, Table S2) and used in an electrophoretic mobility shift assay (EMSA) with increasing concentrations of phosphorylated OmpR (OmpR-P). A PCR-amplified 304-bp fragment of 16S rDNA was included in each binding reaction as a negative control (Fig. 5C, Table S2). Specific OmpR-P binding caused a shift in the migration of the 392 bp fragment F1 that encompasses the OmpR-binding site indicated by *in silico* analysis (Fig. 5C). OmpR-P was unable to bind the control 16S rDNA fragment. Moreover, OmpR-P did not shift the migration of either the upstream regulatory region fragment F2 or the downstream fragment F3, both of which lack the 20 bp OmpR-binding site (Fig. 5C), implying that OmpR binds at the predicted position in fragment F1.

These results demonstrated that OmpR can specifically bind to the *yadA* promoter region, which suggests that expression is inhibited by a direct mechanism. In conclusion, our genetic studies identified *yadA* as a new member of the OmpR regulon. OmpR may modulate the production of YadA in response to environmental signals experienced by *Y. enterocolitica* in different niches during the infection process. Downregulation of YadA might enhance the survival of *Y. enterocolitica* by preventing binding of the bacteria to host cells, thus favouring further dissemination to deeper tissues.

OmpR-dependent production of proteins involved in iron homeostasis

Another group of OmpR-dependent proteins identified in our proteomic analysis belong to the 'Iron ion homeostasis' GO category (Table 1). Proteins of this category included three OM active transporters (also called TonB-dependent transporters): HemR, a receptor involved in heme/haemoprotein uptake (Stojiljkovic and Hantke, 1992); FepA, an iron-enterobactin receptor; and the FecA receptor responsible for dicitrate-mediated iron assimilation (Andrews *et al.*, 2003). These proteins were described previously as iron-regulated receptors whose expression is controlled by the regulator Fur in *Yersinia* spp. (Jacobi *et al.*, 2001; Gao *et al.*, 2008). When the intracellular iron concentration is high, Fur binds iron (Fe^{2+}-Fur) and represses the expression of genes involved in iron/heme acquisition and transport (Hantke, 2001; Troxell and Hassan, 2013). Both FecA and HemR were more abundant (twofold increase) in the *ompR* mutant than in wild-type cells. FecA was affected under almost all conditions, while differences in the level of HemR were detected mainly at 37°C (Table 1). On the other hand, the level of the receptor FepA was slightly decreased in the *ompR* mutant cells (~ 1.5-fold), but only when cultured at 26°C in standard LB medium. The impact of OmpR on receptors of the siderophore and heme uptake systems underscores the role of this regulator in the iron metabolism of *Y. enterocolitica*. Putative OmpR-binding sites were identified in the promoters of the genes *hemR*, *fepA* and *fecA* (Fig. 2).

Insights into the role of OmpR in the repression of hemR

Given our long-standing interest in *Y. enterocolitica hemR*, we further investigated the relationship between OmpR

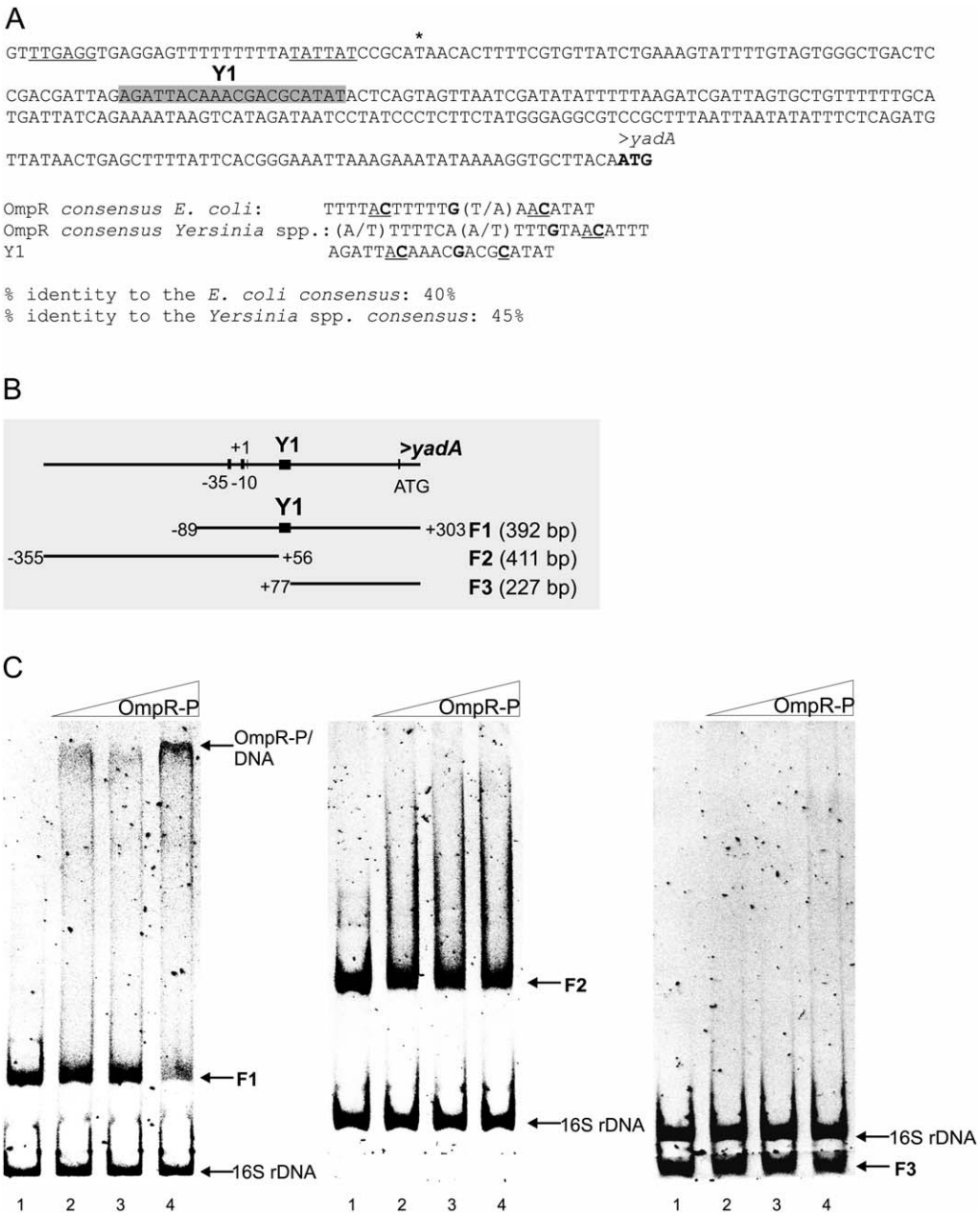


Fig. 5. Interaction of OmpR with the *yadA* promoter region. (A) The promoter and 5′UTR of *yadA*. The experimentally verified −35 and −10 promoter elements (underlined) and the transcription start (asterisk) are indicated (Skurnik and Wolf-Watz, 1989). The sequence shaded grey (Y1) corresponds to the putative OmpR-binding site. The *yadA* start codon (ATG) is shown in bold. Beneath the sequence, the putative binding site Y1 is compared with the consensus OmpR-binding motifs of *E. coli* and *Yersinia* spp. The percentage identity to these sequences is shown. (B) Schematic representation of the *yadA* regulatory region showing the putative OmpR-binding site (Y1) revealed by *in silico* analysis and the position of the DNA fragments (F1, F2, F3) used in electrophoretic mobility shift assays (EMSAs) (C). EMSAs examining the binding of various concentrations of phosphorylated OmpR to fragments of the *yadA* regulatory region: F1 (392 bp), which contains the putative OmpR-binding site, and F2 (411 bp) and F3 (227 bp), which lack this site. A fragment of 16S rDNA (304 bp) was included in each reaction mixture as a non-specific binding control. The binding reactions comprised the DNA fragments mixed with increasing concentrations of OmpR-P (0.168, 0.336, 0.504 μM; lanes 2–4), or with no added OmpR (lane 1). The identities of the bands resolved by electrophoresis on 5% native polyacrylamide gels are indicated.

and HemR. HemR is a unique OM receptor in *Y. enterocolitica*, which can bind heme or multiple host haemoproteins (haemoglobin, haemoglobin-haptoglobin, heme-haemopexin, heme-albumin, myoglobin) (Bracken *et al*., 1999; Runyen-Janecky, 2013). Following binding to HemR, heme is transported through the periplasm and across the IM via the TonB/ExbB/ExbD transport system (Stojiljkovic and Hantke, 1992). The regulation of *hemR*

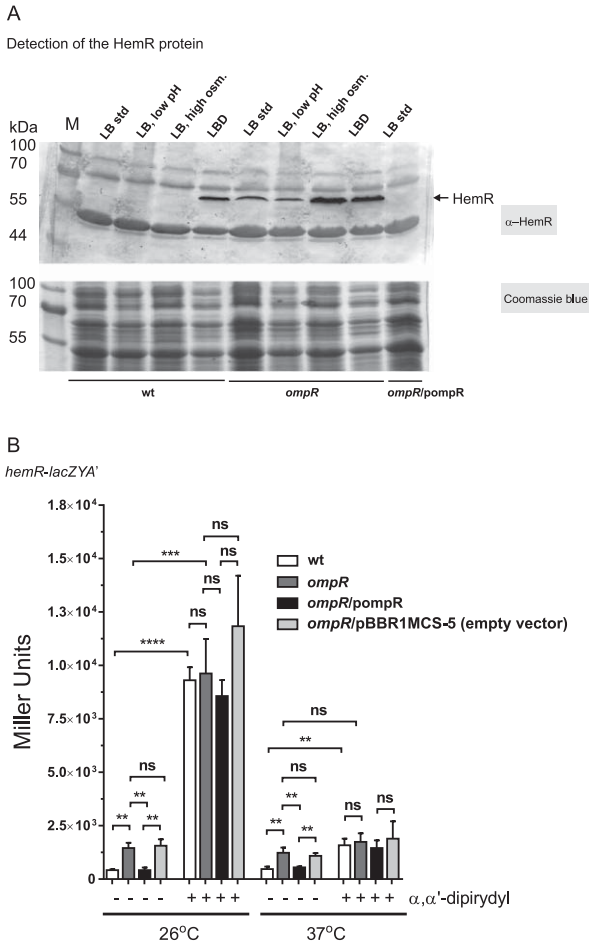


Fig. 6. HemR expression in the wild-type (Ye9) and the *ompR* mutant (AR4) strains.
A. Immunodetection of the HemR protein in total cell extracts of *Y. enterocolitica*. The top panel shows the immunoblot probed with a polyclonal antibody against HemR (α-HemR), and the bottom panel shows the Coomassie blue-stained gel as a loading control. Wild-type strain Ye9 (wt), OmpR-deficient mutant AR4 (*ompR*) and AR4 complemented with a plasmid expressing OmpR (*ompR*/pompR) were grown overnight in LB medium at 37°C, then subcultured in LB medium (standard conditions, std), LB at pH 5.0 (LB, low pH), LB with 386 mM NaCl (LB, high osm.), or LB with 0.3 mM α,α′-dipyridyl (LBD), and incubated at 37°C for 3 h. Equivalent whole-cell lysate samples were loaded. The arrow indicates the HemR band, which is only visible in the parental strain grown under low iron conditions (LBD), but is detected in the *ompR* mutant under all tested growth conditions. M – molecular weight standards (PageRuler Prestained Protein Ladder; kDa). This experiment was repeated twice with similar results.
B. Analysis of *hemR* expression by measuring the β-galactosidase activity of strains carrying a chromosomal *hemR-lacZYA′* transcriptional fusion: wild-type Ye9 (Ye9H), *ompR* mutant AR4 (AR4H), complemented strain AR4H (AR4H/pompR) and AR4H transformed with empty vector pBBR1MCS-5 (AR4H/pBBR1MCS-5). All strains were grown to logarithmic phase in LB medium, with or without 0.3 mM α,α′-dipyridyl, at 26°C or 37°C, and β-galactosidase activity was assayed. The data represent mean activity values (Miller units) with the standard deviation from three independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using Student's unpaired *t*-test (*****P* < 0.0001, ****P* < 0.001, ***P* < 0.01, ns – *P* > 0.05).

expression *in vitro* and *in vivo* was previously elucidated in our laboratory using mouse-virulent *Y. enterocolitica* bio/serotype 1B/O:8 (Jacobi *et al*., 2001). However, the role of the OmpR regulator in the control of *hemR* expression has never been investigated. Since our proteomic analysis showed that the level of HemR receptor is elevated in the OM of the *ompR* mutant strain at 37°C, we first attempted to verify this result by Western blotting using an antibody specific for HemR (Fig. 6A). Parental strain Ye9 and the *ompR* mutant AR4 were grown at 37°C in standard LB medium, LB medium at high osmolarity (386 mM NaCl) or low pH (pH 5.0). As expected, HemR was not visible in the wild-type Ye9 grown in LB medium, and could only be detected in this strain following growth under iron-derepressed conditions (LB with α,α′-dipyridyl, LBD). This result confirmed the iron-regulated status of HemR in agreement with the previously established Fur-mediated repression of the *Y. enterocolitica hemR* gene (Stojiljkovic and Hantke, 1992). In contrast to the wild-type, HemR was detected in the *ompR* mutant AR4 grown in standard LB (under iron-repressed conditions), suggesting that the production of HemR is derepressed in the strain lacking OmpR (Fig. 6A). When the wild-type allele of *ompR* was introduced into mutant AR4 *in trans* on plasmid pompR, the production of HemR in LB medium decreased to the wild-type level, i.e. it was no longer detectable (Fig. 6A). Moreover, HemR was upregulated in the *ompR* mutant in all tested conditions, even in low iron medium (LBD) (Fig. 6A). Interestingly, in the *ompR* mutant grown in LB under high osmolarity conditions (386 mM NaCl), HemR was more abundant than in the same strain grown in standard LB (Fig. 6A). This effect was not observed in LB at low pH. This finding might indicate that in the absence of OmpR another regulatory mechanism operates to increase the HemR level in response to high osmolarity.

We next tested whether the expression of *hemR* is under the control of OmpR (Fig. 6B) by constructing *hemR-lacZYA′* chromosomal transcriptional fusions in the wild-type strain and the *ompR* mutant derivative (strains Ye9H and AR4H respectively). Based on measurements of β-galactosidase activity, we found higher *hemR* expression in strain Ye9H grown in LBD (under iron-starvation conditions) than in LB medium at 26°C (~ 22-fold) and at 37°C (~ 3-fold), confirming the iron-repressible nature of the *hemR* promoter (Fig. 6B). In the *ompR* mutant AR4H, *hemR* expression was upregulated two- to threefold in standard LB medium compared with the wild-type strain Ye9H. This upregulation still occurred in the mutant strain transformed with vector pBBR1MCS-5, but was absent following complementation with the wild-type *ompR* allele on plasmid pompR (Fig. 6B).

Increased *hemR* expression in the *ompR* mutant grown in LB (repressed conditions) might be caused by derepression of *hemR* expression directly and/or by the

alleviation of transcriptional repression by the iron-responsive repressor Fur. To separate these two effects, we examined OmpR-mediated regulation of *hemR* expression under derepressed conditions (released from Fur repression in LBD) at 26°C and 37°C (Fig. 6B). The absence of iron in the medium resulted in an increase in the expression of *hemR* in the *ompR* mutant AR4H, almost to the wild-type level, i.e. OmpR-dependent regulation of *hemR* is lost. This finding suggested that OmpR could regulate *hemR* indirectly, presumably through an effect on *fur* expression.

Interestingly, while the OmpR-dependent regulation of *hemR* transcription disappeared under derepressed conditions (LBD medium), the effect of OmpR on the HemR protein (as judged by immunoblotting) was still observed, suggesting the involvement of OmpR in post-transcriptional regulation of *hemR*. Intriguingly, in *E. coli*, OmpR activates the expression of two small RNAs, OmrA and OmrB, which repress several iron receptor genes (*fepA*, *fecA* and *cirA*) (Guillier and Gottesman, 2006). Only the sRNA OmrA is present in *Y. enterocolitica*, and as in *E. coli* its expression is positively regulated by OmpR (K. Brzostek, unpubl. obs.). Future studies will investigate the role of OmrA in post-transcriptional regulation of iron/heme receptors in *Y. enterocolitica*.

To test whether OmpR directly and/or indirectly regulates *hemR* transcription, we examined the ability of OmpR to bind to the *hemR* promoter region *in vitro*. Previous reports have shown that the *hemR* ORF is located downstream of the *hemP* ORF and that the expression of *hemR* is repressed by iron, suggesting that it is regulated by Fur (Stojiljkovic and Hantke, 1992; Jacobi *et al*., 2001). Using BPROM software, we identified two putative promoters for the *Y. enterocolitica hemR* gene (Fig. 7A). The first is located upstream of the *hemP* ORF and might govern expression of both *hemP* and *hemR*. A well conserved Fur box was identified 412 nucleotides from the beginning of the HemR coding region (Stojiljkovic and Hantke, 1992). The second possible *hemR* promoter is located upstream of the *hemR* ORF. One potential OmpR-binding site (H1, located between nucleotides −179 and −19 bp upstream of the *hemR* ATG) was recognized in this second potential promoter region. This 20 bp element contains the conserved **G**XXA**C** motif, but it exhibits only 30% identity to the *E. coli* and *Yersinia* spp. consensus OmpR-binding site sequences.

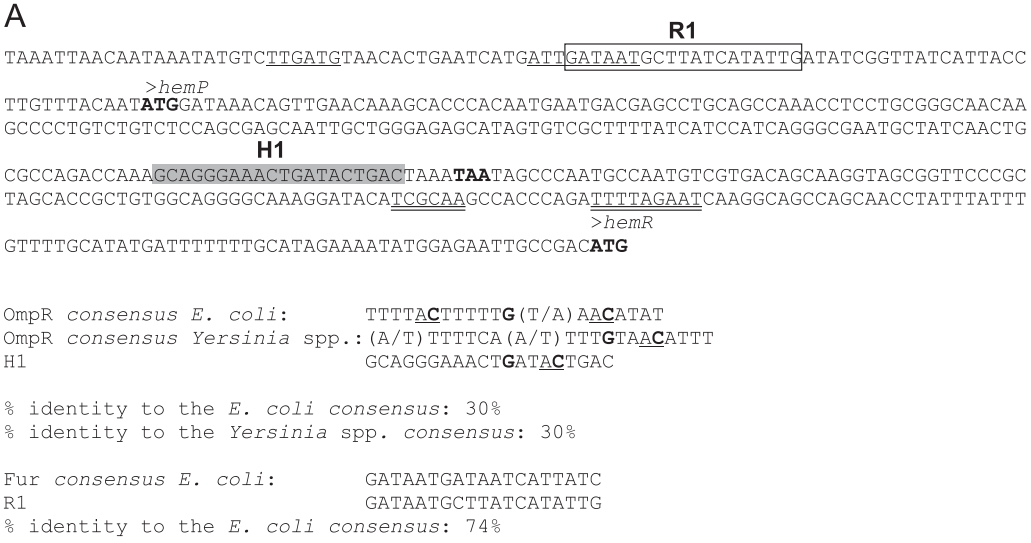
The binding of OmpR to the second putative promoter region of *hemR* was examined in an EMSA (Fig. 7B). Different amounts of phosphorylated OmpR (OmpR-P) were incubated with a 385 bp DNA fragment of the *hemR* gene containing the predicted OmpR-binding site. As shown in Fig. 7B, OmpR-P was unable to bind the putative regulatory region of *hemR*. This result suggested that OmpR indirectly regulates the transcription of *hemR*. Based on our findings, we hypothesize that OmpR might cause repression of *hemR* expression indirectly by its positive influence on Fur expression. Consistent with this hypothesis, four putative OmpR-binding sites were identified in the *fur* regulatory region by *in silico* analysis (data not shown). Detailed studies on the OmpR-dependent regulation of the *fur* gene are currently being performed to verify this hypothesis.

The results of our proteomic analysis raised questions concerning the adaptive role of OmpR associated with the modulation of iron/heme receptor levels. *Yersinia enterocolitica* exhibits a dual lifestyle, existing as both a non-pathogenic saprophyte and a pathogen residing inside the host body. The localization influences the nature of the iron available as well as its dedicated transport mechanisms. In the saprophytic lifestyle, *Y. enterocolitica* may exploit receptors for iron-bound siderophores to acquire iron from the surrounding environment. In the host tissues the majority of iron is found within the heme molecule (free or in haemoproteins). The acquisition of heme by *Y. enterocolitica* occurs via a dedicated HemR-based heme transport system. Thus, the OmpR-mediated regulation of the appropriate OM receptors for iron/heme uptake, according to the local environment, may contribute to the fitness of *Y. enterocolitica*. In particular, regulation of the HemR receptor of the heme transport system by OmpR may be necessary to permit growth of *Y. enterocolitica* within the host. The tight negative regulation of HemR may prevent the acquisition of an excess of heme, which is toxic for bacteria (Anzaldi and Skaar, 2010). Finally, the regulation of the heme uptake system influences cellular levels of the heme moiety. Heme is the prosthetic group of cytochromes and catalase, and an essential cofactor for cellular respiration. Thus, the cellular level of heme may influence respiratory pathways and contribute to changes in the central metabolism of *Y. enterocolitica*. This regulatory network is likely to be significant for other Yersiniae and members of the family Enterobacteriaceae that possess both the response regulator OmpR and a heme transport system based on homologues of HemR (Runyen-Janecky, 2013).

Conclusions

This study represents the first to examine the impact of high osmolarity and low pH on the proteome of *Y. enterocolitica*, and most significantly constitutes the first proteomic analysis of the role of OmpR in this pathogen. Our results indicate that OmpR influences the production of a number of membrane proteins involved in the uptake and transport of compounds into the cell and in efflux or secretion processes. Thus, OmpR may have an impact on the passage of solutes across the cell envelope

A



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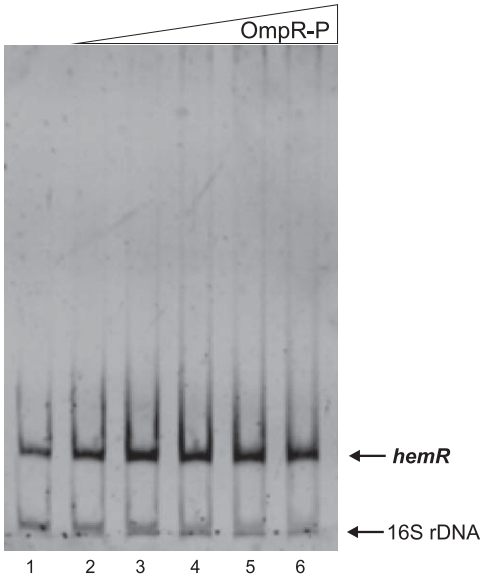

Fig. 7. Interaction of OmpR with the *hemR* promoter region.
A. The *hemPR* and *hemR* promoters and 5′UTRs. The putative −35 and −10 promoter elements of *hemPR* and *hemR* are single and double underlined respectively. The Fur binding site in the *hemP* ORF is boxed (R1). The sequence shaded grey (H1) corresponds to the putative OmpR-binding site. The start codons (ATG) of *hemP* and *hemR*, and the stop codon of *hemP*, are shown in bold (Stojiljkovic and Hantke, 1992; Thompson *et al*., 1999). Beneath the sequence, the putative OmpR and Fur binding sites are compared with the respective consensus binding motifs, and the percentage identities are shown.
B. Electrophoretic mobility shift assay of a *Y. enterocolitica hemR* promoter region fragment (385 bp) incubated with purified and *in vitro* phosphorylated OmpR protein. A fragment of 16S rDNA (304 bp) was included as a non-specific binding control. The binding reactions comprised the DNA fragments mixed with increasing concentrations of OmpR-P [0.38, 0.76, 1.14, 1.52, 3.04 μM (lanes 2–6) or with no added OmpR (lane 1)]. The identities of the bands resolved by electrophoresis on 5% native polyacrylamide gels are indicated.

when Yersiniae are exposed to the varied environmental conditions associated with different ecological niches. Moreover, OmpR appears to influence *Y. enterocolitica* pathogenesis by (i) modulating the expression of proteins that are likely to promote cellular survival in acidic pH and (ii) repressing the expression of adhesin YadA, a major virulence factor. Finally, our results provide some novel insights into the role of OmpR in the remodelling of the bacterial surface, a vital strategy associated with growth/survival in niches outside and within the host organism, which vary in osmolarity, pH and iron/heme content. These findings identify OmpR as the central integrator of several cellular processes regulating the dual saprophytic and pathogenic lifestyles of *Y. enterocolitica*.

Experimental procedures

Bacterial strains and growth conditions

The strains and plasmids used in this study are listed in Table S1. Unless indicated, *Y. enterocolitica* strains were cultured at 26°C in LB medium. *Escherichia coli* strains were grown at 37°C in LB medium. As required, media were supplemented with the appropriate antibiotics: nalidixic acid (Nal) – 30 μg ml^{-1}, chloramphenicol (Cm) – 25 μg ml^{-1}, kanamycin (Km) – 50 μg ml^{-1}, gentamicin (Gm) – 40 μg ml^{-1}, spectinomycin (Sp) – 100 μg ml^{-1}. For iron-derepressed growth conditions, Yersiniae strains were cultured in LB medium supplemented with 0.3 mM α,α′-dipyridyl to chelate iron ions (LBD medium). For proteomic experiments, triplicate overnight cultures of *Y. enterocolitica* strains Ye9 and AR4 were grown in LB, pH 7.0 at 26°C or 37°C to an OD_{600} of 1.0–1.3. The cultures were then centrifuged (5000 × *g*, 10 min) and the cells re-suspended to an OD_{600} of 1.0 in 25 ml of (i) fresh LB at pH 7.0 with 86 mM NaCl (standard medium), (ii) LB adjusted to pH 5.0 by the addition of 100 mM HOMOPIPES buffer [homopiperazine-N,N′-bis-2-(ethanesulfonic acid)], or (iii) LB at pH 7.0 supplemented with NaCl to 386 mM. The pH of all LB media was measured and found not to change significantly during subsequent growth of the cells. Replicate cultures were incubated at 26°C or 37°C with shaking for 3 h, then 25 ml samples were centrifuged (8000 × *g*, 20 min, 4°C), and the cell pellets flash frozen in liquid nitrogen and stored at −80°C prior to fractionation for proteomic analysis.

Isolation of outer membrane-enriched sarkosyl-insoluble fractions for shotgun label-free quantitative proteomic analysis

Each of the triplicate bacterial pellets from the different culture variants (36 samples, i.e. 2 strains × 2 temperatures × 3 media × 3 biological replicates) was re-suspended in half the original culture volume of buffer (200 mM Tris HCl pH 8.0, 0.5 M sucrose, 250 μg ml^{-1} lysozyme, 1 mM EDTA), incubated for 1 h at 4°C and sonicated on ice for 18 cycles of 30 s, separated by 30 s intervals, using a Sonics Vibra-Cell VCX 130 (Sonics & Materials, Newtown, CT, USA). After centrifugation (8000 × *g*, 10 min, 4°C) to remove unbroken cells and debris, the supernatants were centrifuged at high speed (35 000 × *g*, 1.5 h, 4°C) to pellet total membranes. Membrane pellets were then re-suspended in 10 ml of 2% sodium lauroyl sarcosine (sarkosyl) in 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4 and incubated for 1 h at 37°C, with occasional shaking to solubilize the IM (Filip *et al*., 1973). The sarkosyl-insoluble outer membrane-enriched (OMsl) fractions were pelleted by high-speed centrifugation as described above.

Mass spectrometry

Sarkosyl-insoluble OM pellets were suspended in 40 μl of SDS/deoxycholate buffer (0.1% SDS, 1% sodium deoxycholate, 20 mM DTT, 100 mM Tris HCl pH 8.5, in 25 mM ammonium bicarbonate) and sonicated in a water bath (20 cycles of 30 s) to solubilize the OM proteins. After clarification by centrifugation (15 min, 12 000 × *g*), protein concentrations in the supernatant samples were estimated using a BCA assay (Pierce) and normalized by dilution in 25 mM ammonium bicarbonate solution. The proteins were then reduced by treatment with 50 mM DTT (30 min, 60°C), alkylated with 80 mM iodoacetic acid (45 min at room temperature – RT) and 50 μg samples were digested overnight with trypsin (sequencing Grade Modified Trypsin; Promega V5111).

The digestion reactions were quenched by acidifying the samples with 0.6% TFA (trifluoroacetic acid) and precipitated sodium deoxycholate was removed by centrifugation. Each peptide mixture was applied in turn to a RP-18 precolumn (nanoACQUITY Symmetry® C18, Waters 186003514), using 0.1% TFA in water as the mobile phase, and then to a nano-HPLC RP-18 column (nanoACQUITY BEH C18, Waters 186003545) using an acetonitrile gradient (5–35% AcN over 180 min) in the presence of 0.05% formic acid, with a flow rate of 250 nl min^{-1}. The column outlet was directly coupled to the ion source of an Orbitrap Velos mass spectrometer (Thermo Electron Corp, San Jose, CA) working in the regime of data-dependent MS to MS/MS switch. To prevent cross contamination by previous samples, each analysis was preceded by a blank run. The raw data were processed using Mascot Distiller followed by Mascot Search (Matrix Science, London, UK) to identify hits in the Swiss-Prot database (20110124) restricted to *Yersinia* sequences. The following search parameters were applied: precursor and product ion mass tolerances – 20 ppm and 0.4 Da, respectively; enzyme specificity of trypsin – 1 missed cleavage site allowed; permitted modifications – cysteine carbamidomethylation and methionine oxidation. To estimate the false-positive discovery rate (FDR), the decoy search option was enabled. Peptides with a Mascot Score exceeding the threshold value corresponding to < 1% FDR were considered to be positively identified. Label-free quantitation was performed as described previously (Bakun *et al*., 2012; Malinowska *et al*., 2012). Briefly, both qualitative and quantitative runs were performed for each sample. From the qualitative run, peptide sequences, masses and retention times were obtained, while from the quantitative run, peptide masses, retention times and intensities were acquired. We used MS1 peak integration to obtain the intensities of individual peptides. The protein intensity ratio between two groups or samples was calculated as the median of the intensity ratios for all its peptides where the quantitative values are not missing. Using an in-house software pipeline, data from these two measurements were integrated, resulting in a list of identified peptides and their intensities for a given sample. These lists were then subjected to statistical analysis using in-house DIFFPROT software to identify differentially expressed proteins. Details of the subsequent bioinformatic analyses are provided below.

Bioinformatic analyses

Following mass spectrometry, protein lists were generated by Mascot and further filtered using in-house MSCAN software to select proteins with an FDR of < 1%, identified by at least two peptides. The obtained shortlist of selected peptides (SPL) was used to tag peptide peaks in 2D heat-maps generated on the basis of the MS profile data. In detail, LC-MS data

obtained directly from the mass spectrometer were converted into 2D heat maps using an in-house MsConvert data conversion tool. This file format is recognized by Msparky, an in-house modification of the commonly used graphical NMR assignment and integration programme Sparky NMR (http://www.cgl.ucsf.edu/home/sparky). Msparky displays LC-MS data as 2D peptide heat-maps (with peptide LC Rt and m/z as the vertical and horizontal axes respectively). Overlaying qualitative data (SPL) on quantitative profile datasets (2D heat maps) was performed by Msparky, which matches the sequence information with intensity data for peptide signals of the same m/z and LC Rt, on the basis of m/z, Rt and isotopic profile fitting. The automatic labelling of peptide signals provided by Msparky was verified by manual data inspection, applying the following acceptance criteria: m/z value deviation – 20 ppm; LC retention time deviation – 10 min; envelope root mean squared error (deviation between the expected isotopic envelope of the peak heights and their experimental values) – 0.6. Qualitative and quantitative data were integrated and subjected to statistical analysis using in-house Diffprot software. Statistical significance values were calculated using the re-sampling test implemented in Diffprot (Malinowska *et al*., 2012). Diffprot processes obtained peptides lists by clustering proteins into families, removal of non-unique peptides and signal intensities normalization with Lowess method. In the next stage, Diffprot employs a re-sampling-based statistics paired with FDR procedure for estimating the statistical significance of quantitative results, as well as a local-pooled-error-like procedure to deal with small number of biological replicates. Results are displayed in a table format with proteins segregated according to statistical relevance, supported by information on how many peptides were used for the analysis and observed ratio. All software used is accessible at http://proteom.ibb.waw.pl.

We ran our searches against entire *Yersinia* genus database, which contains 479355 sequences. Most identified peptides matched multiple protein sequences. To remove redundant orthologues, we grouped protein sequences with highly similar sets of identified peptides (at least 90% cluster-coverage identical peptide sequences) into clusters, and then assigned peptide-spectrum matches (PSMs) to sequence clusters, removing those with no unique assignment. Next we mapped each cluster to accession number of one of its members, preferably a sequence from our reference strain *Y. enterocolitica* subsp. *palearctica* 105.5R(r). If that has not been possible the other strains (i.e. *Y. enterocolitica* subsp. *palearctica* Y11), other subspecies (i.e. *Y. enterocolitica* subsp. *enterocolitica* 8081) or other species were chosen, in order.

Other bioinformatic analyses were based on the complete genome sequences of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) and *Y. enterocolitica* subsp. *enterocolitica* 8081 (GenBank; http://www.ncbi.nlm.nih.gov/genbank/). Gene Ontology data were obtained from the UniProt databases (http://www.uniprot.org). Principal component analysis was performed using in-house software produced with scikit-learn (http://scikit-learn.org). Promoter prediction was conducted using the web-based software BPROM in the Softberry package (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb; Solovyev and Salamov, 2011). Logo motif analysis to identify potential OmpR-binding sites within promoter regions was performed using WebLogo (Crooks and Hon, 2004; http://weblogo.berkeley.edu/logo.cgi).

Molecular biology techniques

All DNA manipulations, including PCR, restriction digestions, ligations and DNA electrophoresis, were performed as previously described (Sambrook and Russell, 2001). Plasmid and chromosomal DNA were isolated using a Plasmid Miniprep DNA Purification Kit and Bacterial & Yeast Genomic DNA Purification Kit respectively (EURx, Gdańsk, Poland). Restriction enzymes were obtained from Thermo Scientific (Waltham, USA). Polymerase chain reaction was routinely performed in 25 μl or 50 μl reaction mixtures for 35 cycles using Taq DNA polymerase or, when fragments were used for cloning, Phusion High-Fidelity DNA Polymerase (Thermo Scientific). DNA fragments amplified by PCR were purified with a PCR/DNA Clean-Up Purification Kit (EURx) before and after restriction digestion. All kits and reagents were used according to the recommendations of the supplier. Oligonucleotide primers used for PCR and sequencing were purchased from Genomed S.A. (Warsaw, Poland) and are listed in Table S2. Plasmids used in this study are described in Table S1. DNA sequencing was performed by Genomed S.A.

Western blotting

The abundance of selected proteins in *Y. enterocolitica* cells was evaluated by Western blotting using the OMsl fractions prepared as for proteomic analysis (YadA) or total bacterial protein extracts (HemR). The final protein concentrations in the OMsl samples were estimated using the RC-DC protein assay (Bio-Rad, Hercules, USA) and normalized by dilution in Laemmli buffer (Sambrook and Russell, 2001). For detection of YadA in the oligomeric form, the OMsl samples were re-suspended in Laemmli buffer and boiled for 5 min prior to electrophoresis. For the detection of YadA in the form of monomers, the samples were re-suspended in urea sample buffer (62.5 mM Tris/HCl, pH 6.8, 8 M urea, 10% glycerol, 2% SDS, 0.00125% bromophenol blue) and boiled for 10 min. To prepare total protein extracts for HemR analysis, the cultures were normalized to the same OD_{600}, and after centrifugation the cell pellets were re-suspended in Laemmli buffer and boiled for 5 min prior to electrophoresis. Equivalent samples were separated on 8% (for HemR) or 10% (for YadA) polyacrylamide gels by electrophoresis (SDS-PAGE), then transferred to nitrocellulose membrane (Amersham Protran Western blotting membrane, pore size 0.2 μM; GE Healthcare) using a wet electroblotting system (Bio-Rad). The blots were probed with rabbit antisera directed against HemR (1:8000) or YadA (1:5000). Both polyclonal antibodies were prepared at the Max von Pettenkofer Institute for Hygiene and Medical Microbiology (University of Munich). Goat anti-rabbit IgG, conjugated to alkaline phosphatase (Sigma-Aldrich, St Louis, USA), was used as the secondary antibody (diluted 1:30 000). Positive immunoreaction was visualized using the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT; Sigma-Aldrich). In each experiment, the loading

of equivalent amounts of protein was controlled by Coomassie blue staining of an identical gel.

Construction of transcriptional hemR-lacZYA′ reporter fusion

To construct a *hemR* promoter-*lacZYA′* fusion, a 385 bp fragment of the *hemR* promoter region was amplified from Ye9 chromosomal DNA using primers HemR1 and HemR2 (Table S2). The product was initially cloned into the cloning vector pDrive (Qiagen, Venlo, Netherlands), and then, following digestion with XbaI/SmaI, the released insert was subcloned into suicide plasmid pFUSE cleaved with the same enzymes to place them immediately upstream of a promoterless β-galactosidase gene (Baumler *et al*., 1996). The suicide vector construct containing the *hemR* fragment, verified by restriction digestion and DNA sequencing, was named pFH. This plasmid was used to transform *E. coli* S17 λ*pir* and then introduced into *Y. enterocolitica* Ye9N and the OmpR-deficient mutant strain AR4 by biparental mating. Because pFUSE cannot replicate in *Y. enterocolitica* cells, all selected transconjugants carried the plasmid integrated into the genome. The conjugation between the donor and recipient strains was performed on LB agar plates for 18 h at RT. The Ye9N exconjugants were selected on LB agar plates containing chloramphenicol (25 μg ml^{-1}) and nalidixic acid (30 μg ml^{-1}), and the AR4 exconjugants on LB containing chloramphenicol (25 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). Single-crossover homologous recombination yielded genomic transcriptional fusion between the *hemR* promoter and the promoterless *lacZYA′* operon. The correct insertion of the suicide vector was verified by PCR using one primer (HemR3) located upstream of the homologous region used for recombination and another primer (lacZH991) within the *lacZ* gene, followed by sequencing of the amplified product. Strains carrying the desired transcriptional fusions were designated AR4H and Ye9H (*hemR-lacZYA′*).

Construction of plasmid pompR for complementation

To complement the *ompR* mutation, the *ompR* gene with the native ribosome binding site was amplified by PCR using Ye9 chromosomal DNA as the template with primers OmpB1 and OmpB2 (Table S2). The product was initially cloned into cloning vector pDrive (Qiagen), and then an EcoRI/BamH1 fragment was subcloned into plasmid pBBR1MCS-5 cleaved with the same enzymes (Kovach *et al*., 1995). The resulting construct, pompR, was verified by DNA sequencing and used to transform *E. coli* S17 λ*pir*. This plasmid was then introduced into *ompR* mutant strain carrying the transcriptional *lacZYA′* reporter fusion (AR4H) by biparental conjugation. The exconjugants were selected on LB agar plates containing gentamicin (40 μg ml^{-1}) and kanamycin (50 μg ml^{-1}). The parent vector pBBR1MCS-5 was introduced into the same strain as a negative control.

β-galactosidase assays

β-galactosidase assays were performed essentially as described by Thibodeau and colleagues (2004), using 96-well microtiter plates and a Sunrise plate reader (Tecan, Männedorf, Switzerland). Briefly, cultures were grown overnight and were next diluted into subcultures, which were then grown under various conditions in 96-well plates with shaking (250 r.p.m.) to an OD_{600} of 0.3–0.5. 80 μl of each cell suspension was mixed with 10 μl of POPCulture Reagent (EMD Millipore Corp, Billerica, USA) and 4 units of lysozyme (Sigma-Aldrich), then incubated for 15 min to cause lysis. In the wells of a microtiter plate, 20 μl of each cell lysate was mixed with 130 μl Z-Buffer and 30 μl ONPG (4 mg ml^{-1}) as described by Miller (1992). For kinetic assays, the absorbance at 415 nm (relative to a blank) was measured at time intervals of 10 s, with 2 s of shaking before each reading. The assays were performed at 25°C and monitored for up to 20 min. Data were analysed using Magellan data analysis software. The β-galactosidase activity was expressed in Miller units calculated as described previously (Thibodeau *et al*., 2004). Each assay was performed at least in triplicate.

Construction of GFP translational fusions with YadA

To measure transcriptional and post-transcriptional regulation of *yadA* expression, a translational fusion with GFP was constructed in plasmid pFX-P (Schmidtke *et al*., 2013) using the Golden Gate technique (Engler *et al*., 2008). A DNA fragment carrying the promoter, 5′ untranslated region (5'UTR) and the first 16 codons of the *yadA* gene was amplified from Ye9 plasmid pYV DNA by PCR using primers YadA4 and YadA5 (Table S2). These primers contained BsaI sites and additional sequences designed to generate compatible ends with BsaI-cleaved pFX-P (Table S1). In a 20 μl Golden Gate cloning reaction, 40 fmol of vector was mixed with 40 fmol of PCR product, 5 units of BsaI (New England Biolabs, Frankfurt am Main, Germany) and 4.5 units of ligase (Thermo Scientific) in ligase buffer. The reaction was incubated at 37°C for 1 h, 5 min at 50°C, followed by 5 min at 80°C, and then used to transform *E. coli* DH5α by electroporation. The recombinant fusion construct pFX-yadA and negative control plasmid pFX-0 (Schmidtke *et al*., 2013) were introduced into parental and *ompR*-negative *Y. enterocolitica* strains by electroporation.

Monitoring bacterial fluorescence by flow cytometry

Three independent overnight cultures of each strain grown from single colonies in LB medium supplemented with spectinomycin were diluted 1:20 in fresh medium and incubated at 27°C or 37°C. After approximately 22 h, the bacteria were diluted in sterile phosphate-buffered saline to approximately 4–8 × 10^6 CFU ml^{-1}. For every sample, the mean fluorescence intensity of at least 20 000 bacterial cells was measured with a FACS Canto II flow cytometer (BD) using the FITC filter settings and analysed with the FACS Diva Software v6.1.2.

Construction of plasmid pETOmpR

To express OmpR as a fusion protein with an amino-terminal His_6 extension, a 725 bp fragment representing the entire *ompR* coding sequence was amplified from *Y. enterocolitica*

chromosomal DNA with primers OmpRpET1 and OmpRpET2 (Table S2). The PCR product was digested with NheI and SalI and cloned into vector pET28a (Novagen) cleaved with the same enzymes. The resulting construct, pETOmpR, was verified by restriction digestion and sequencing and used to transform *E. coli* BL21(DE3).

Overproduction and purification of OmpR-His$_6$

The N-terminal His-tagged OmpR protein (OmpR-His$_6$, 29.78 kDa) was expressed and purified using Ni-NTA resin (Qiagen) as described in the manufacturer's standard protocol. Briefly, *E. coli* BL21(DE3) carrying plasmid pETOmpR was grown to mid-logarithmic phase, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.8 mM, and the culture incubated for a further 4 h at 37°C. The cells were then pelleted by centrifugation, re-suspended in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, 55 μM PMSF, 5 mM imidazole and 10 mM 2-mercaptoethanol, and disrupted by sonication. After centrifuging the cell lysate to remove unbroken cells, the supernatant was passed through a Ni-NTA agarose column. The column was washed with 5 volumes of 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl, and then bound protein was eluted using a gradient of imidazole buffer. The fractions were analysed by SDS-PAGE, and those containing the purified OmpR-His$_6$ protein were loaded into a Slide-A-Lyzer Dialysis Cassette (10K MWCO; Thermo Scientific) and dialysed at 4°C in 20 mM HEPES (pH 7.9) buffer containing 100 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol (Fernandez-Mora *et al*., 2004). The concentration of the purified OmpR protein was estimated using the RC DC protein assay (Bio-Rad).

EMSAs

The interaction between phosphorylated OmpR protein (OmpR-P) and the promoters of selected genes was examined essentially as described previously (Raczkowska *et al*., 2011a). The primers listed in Table S2 were used in PCRs with *Y. enterocolitica* genomic DNA to amplify fragments comprising the regulatory regions of the genes *yadA* and *hemR*. Purified OmpR-His6 was phosphorylated *in vitro* by incubation for 30 min at RT in phosphorylation buffer [50 mM Tris pH 8.0, 20 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 5% glycerol containing 20 mM acetyl phosphate (lithium potassium acetyl phosphate; Sigma-Aldrich)]. The purified DNA fragments (0.3 pmol in 20 μl) were then incubated with different amounts of OmpR-His$_6$ at RT for 30 min. The reactions were analysed by electrophoresis on 5% native polyacrylamide gels (29:1 acrylamide/bis acrylamide) in 0.5× Tris-borate-EDTA buffer for 0.5 h at 90 V and 2.5 h at 130 V at 4°C. As a negative control, a 304 bp fragment of the *Y. enterocolitica* 16S rRNA gene amplified by PCR (Table S2) was included in the binding reactions. Ethidium bromide (Sigma-Aldrich) was used to stain the DNA bands in the gels, which were visualized on a UV transilluminator.

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Supporting information

Additional Supporting Information may be found in the online version of this article on the publisher's web-site:

Fig. S1. Summary of OmpR-dependent changes in protein production under the different growth conditions tested. Differences in the abundance of proteins in the OMsl of the *ompR* mutant (strain AR4) compared with the wild-type (strain Ye9) were studied in strains grown under standard conditions (LB), high osmolarity (LB supplemented with 386 mM NaCl) or low pH (LB, pH 5.0), at 26°C and 37°C. Significant changes in protein abundance (q-value ≤ 0.05) of ≥ 1.5 were accepted. (A) Venn diagram illustrating the total number of OmpR-dependent changes observed at 26°C and 37°C, and their distribution between standard conditions, high osmolarity and low pH. (B) Venn diagrams showing the extent of the overlap between the proteins differentially expressed (less or more abundant) in the *ompR* mutant AR4 compared with the wild-type strain Ye9 at 26°C (left sets) versus 37°C (right sets) under standard conditions, high osmolarity and low pH.

Fig. S2. Principal component analysis used to cluster the identified protein patterns according to OmpR status and growth conditions. The effect of temperature (26°C versus 37°C), pH (pH 7.0 versus pH 5.0) and osmolarity (86 mM versus 386 mM NaCl) on the wild-type Ye9 and *ompR* mutant AR4 protein patterns is shown (A). Each point corresponds to a single replicate sample. The value of the principal components is not a measure of the magnitude of the variable. PCA is used to cluster the protein patterns at 26°C and 37°C produced by OmpR activity under standard growth conditions (B), high osmolarity (C) and pH 5.0 (D). Each point corresponds to the protein pattern of each replicate sample generated by the presence (wild-type Ye9) or absence (*ompR* mutant AR4) of OmpR under the particular growth conditions, projected onto a two-dimensional principal component space.

Table S1. Strains and plasmids used in this study.

Table S2. Primers used in this study.

Table S3. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C. Differentially expressed proteins identified in the OMsl (outer membrane-enriched sarkosyl-insoluble fractions) are described according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Proteins were clustered based on Gene Ontology (biological process) terms. Significant changes in protein abundance (q-value ≤ 0.05) are defined by a ratio of ≤ 0.67 (protein more abundant at 37°C or at high osm. or low pH) or ≥ 1.5 (protein less abundant at 37°C or at high osm. or low pH). Values for the fold change in abundance and the number of identified peptides belonging to the proteins are indicated.

Table S4. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 and isogenic *ompR* mutant AR4 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C. Differentially expressed proteins identified in the OMsl are described according to their UniProt database or GenBank entries, or their similarity to homologous sequences identified using BLAST searches. Significant changes in protein abundance (q-value ≤ 0.05) are defined by the ratio of ≤ 0.67 (protein more abundant in *ompR* mutant strain) or ≥ 1.5 (protein less abundant in *ompR* mutant strain). Values for the fold change in abundance and the number of identified peptides belonging to the proteins are indicated.

Appendix S1. Detailed description of the effect of temperature, osmolarity and pH on the membrane proteome of the wild-type *Y. enterocolitica* strain Ye9 presented in Table S3.

Supporting Information

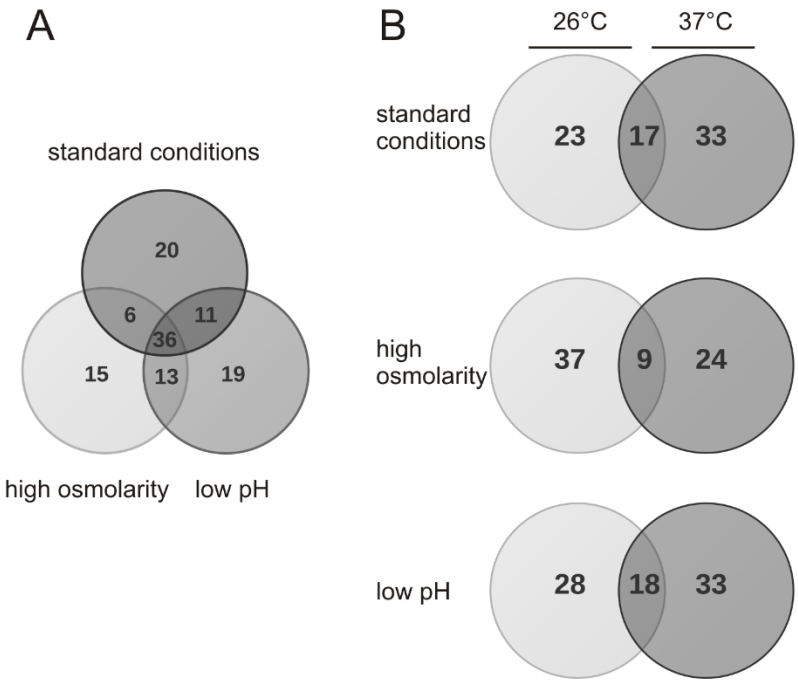

Fig. S1. Summary of OmpR-dependent changes in protein production under the different growth conditions tested. Differences in the abundance of proteins in the OMsl of the *ompR* mutant (strain AR4) compared with the wild-type (strain Ye9) were studied in strains grown under standard conditions (LB), high osmolarity (LB supplemented with 386 mM NaCl) or low pH (LB, pH 5.0), at 26°C and 37°C. Significant changes in protein abundance (*q*-value ≤ 0.05) of ≥ 1.5 were accepted. (A) Venn diagram illustrating the total number of OmpR-dependent changes observed at 26°C and 37°C, and their distribution between standard conditions, high osmolarity and low pH. (B) Venn diagrams showing the extent of the overlap between the proteins differentially expressed (less or more abundant) in the *ompR* mutant AR4 compared with the wild-type strain Ye9 at 26°C (left sets) versus 37°C (right sets) under standard conditions, high osmolarity and low pH.

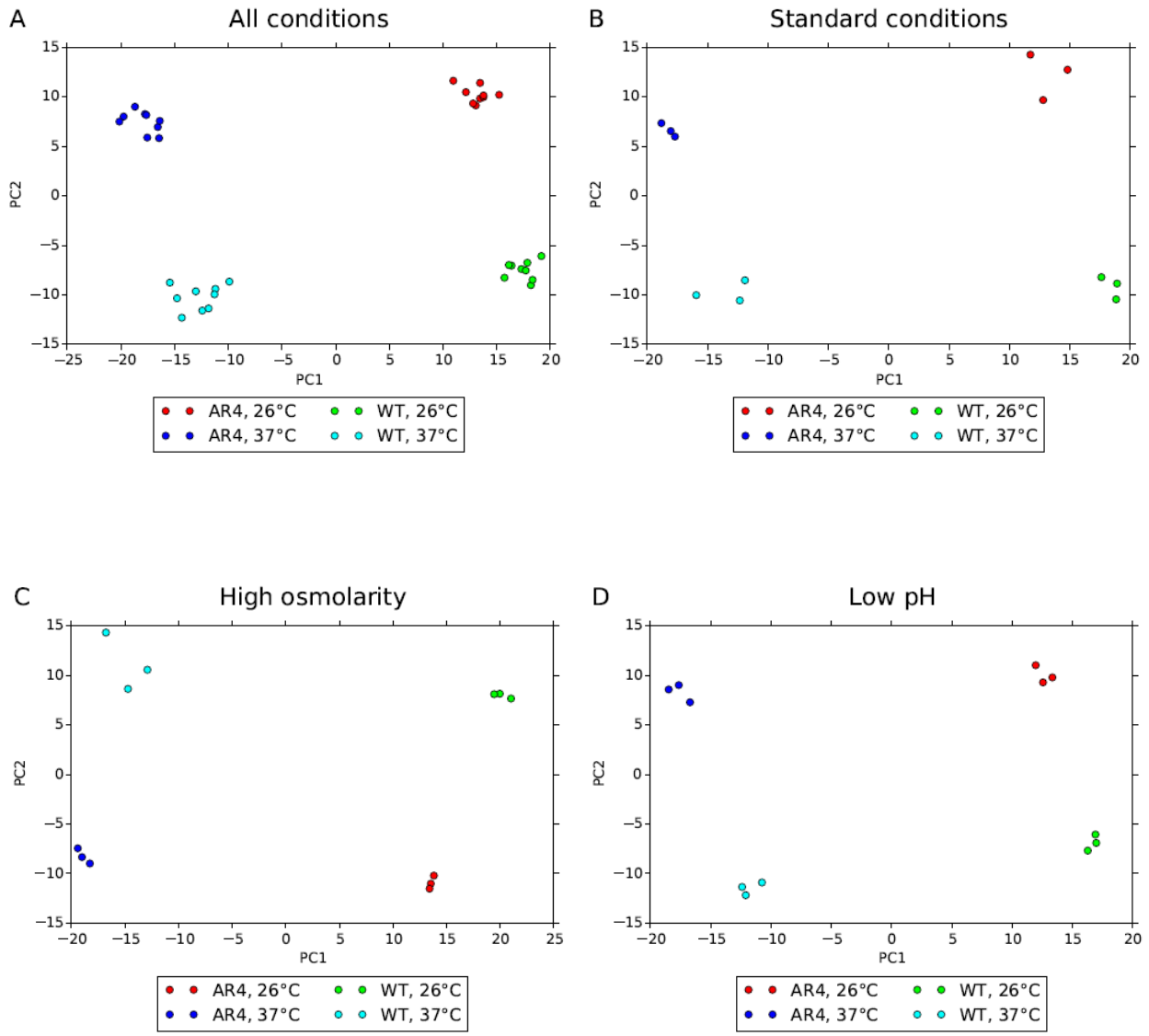

Fig. S2. Principal component analysis used to cluster the identified protein patterns according to OmpR status and growth conditions. The effect of temperature (26°C versus 37°C), pH (pH 7.0 versus pH 5.0) and osmolarity (86 mM versus 386 mM NaCl) on the wild-type Ye9 and *ompR* mutant AR4 protein patterns is shown (A). Each point corresponds to a single replicate sample. The value of the principal components is not a measure of the magnitude of the variable. PCA is used to cluster the protein patterns at 26°C and 37°C produced by OmpR activity under standard growth conditions (B), high osmolarity (C) and pH 5.0 (D). Each point corresponds to the protein pattern of each replicate sample generated by the presence (wild-type Ye9) or absence (*ompR* mutant AR4) of OmpR under the particular growth conditions, projected onto a two-dimensional principal component space.

Supplementary Table S1. Strains and plasmids used in this study.

Strains and plasmids	Description	Reference or source
***Y. enterocolitica* O:9**		
Ye9	wild-type, pYV^+	Clinical isolate, laboratory collection
Ye9N	Ye9, Nal^R, pYV^+	Brzostek et al., 2007
Ye9c	pYV-cured derivative of Ye9	Skorek et al., 2013
Ye9H	Ye9N, *hemR::lacZYA'*, Nal^R, Cm^R	This work
AR4	Ye9N, Δ*ompR*::Km, Nal^R, Km^R, pYV^+	Brzostek et al., 2003
AR4H	AR4, *hemR::lacZYA'*, Km^R, Cm^R	This work
E. coli		
S17-1 λpir	*pro thi recA hsdR514* (R^+M^-) *λpir* RP4 2-Tc::Mu-Kn::Tn7 (Tp^R Str^R)	Simon et al., 1983
TOP10 F'	F' {*lacI*^{q} Tn10 (Tet^R)}*mcrA* Δ(*mrr-hsd RMS-mcrBC*)*φ80lacZ ΔM15 ΔlacX74 deoR recA1 araD139 Δ99ara-leu*)7697*galU galK rpsL* (Sm^R) *endA1 nupG*	Invitrogen
DH5α	F^- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) *U169* rec*A1* end*A1* hsd*R17 (rK*$^-$*, mK*$^+$*)* pho*A* sup*E44 λ*$^-$ *thi*$^-$ *1* gyr*A96* rel*A1*	Sambrook et al., 1989
BL21 (DE3)	*fhuA2 [lon] ompT gal (λ DE3) [dcm]* Δ*hsdS* *λ DE3 = λ sBamHIo* Δ*EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21* Δ*nin5*	Life Technologies
Plasmids		
pDrive	cloning vector, Ap^R, Km^R	Qiagen
pFUSE	suicide vector, derivative of pEP185.2 with promoterless *lacZYA* genes, Cm^R	Baumler et al., 1996
pFH	pFUSE with XbaI/SmaI fragment (385-bp) of *hemR*, Cm^R	This work
pBBR1MCS-5	broad-host-range cloning vector, *ori* pBBR1, Mob^+, oriT RK2, Gm^R	Kovach et al., 1995
pompR	pBBR1MCS-5 carrying *ompR* with RBS (EcoRI/BamHI fragment), Gm^R	This work
pET28a	expression vector with 6His-tag coding sequence, Km^R	Novagen
pETOmpR	pET28a carrying the entire *ompR* coding sequence (725-bp fragment), Km^R	This work
pFX-P	derivative of pDSK602, carries a BsaI-flanked dummy module and *gfp* coding sequence without start codon, compatible with Golden Gate cloning	Schmidtke et al., 2013
pFX-yadA	pFX-P derivative carrying 686 bp upstream of *yadA* start codon and the first 16 codons of *yadA* fused to *gfp*, Sp^R	This work
pFX-0	pFX-P derivative, entire *gfp* ORF without promoter, Sp^R	Schmidtke et al., 2013

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Supplementary Table S2. Oligonucleotide primers used in this study.

Name of primer	Primer sequence (5'→ 3')*	Restriction enzyme	Purpose
HemR1 (F)	**GATCTAGA**CAGTTGAACAAAGCACCCAC	XbaI	Transcriptional fusion and EMSA
HemR2 (R)	**TACCCGGG**ACGGAAACGGTCGGAAGT	SmaI	
HemR3 (F)	CCTGGTTACTCGGGAAGATG		Verifying correctness of the fusion
lacZH991 (R)	CATCGCAGGCTTCTGCTTC		Verifying correctness of the fusion
YadA4 (F)	**TTTGGTCTC**TTAGCCGCAGATATTAATGCCGCAG	BsaI	Translational fusion
YadA5 (R)	**TTTGGTCTC**TATTCCCGTTTATGGTTCCAGACA	BsaI	
OmpB1 (F)	**TGGAATTC**CAATACGGCCTTTGGGAGTA	EcoRI	Cloning
OmpB2 (R)	**TGGGATCC**CGGCATTCACCACCATATTT	BamHI	
OmpRpET1 (F)	**CTAGCTAGC**ATGCAAGAGAATCACAAGATTCTG	NheI	Cloning
OmpRpET2 (R)	**ACGCGTCGA**CTCATGCTTTACTGCCG	SalI	
YadA7 (F)	GGTGAAAGTAAATGTGTTATCAGGTAAT		EMSA, F1fragment
YadA6 (R)	TAATGCCGCAGAGACACTGA		
YadA8 (F)	TGGATAACGCTCGATCACTG		EMSA, F2 fragment
YadA9 (R)	CTAATCGTCGGAGTCAGCCC		
YadA10 (F)	ACTCAGTAGTTAATCGATATATTTTTAAGATCG		EMSA, F3 fragment
YadA11 (R)	TAATGCCGCAGAGACACTGA		
16SR1 (F)	ATTCCGATTAACGCTTGCAC		EMSA, 16S rDNA
16SR304 (R)	GTGGGGTAATGGCTCACCTA		

* 5' extensions added to introduce cleavage sites for the indicated restriction enzymes are shown in bold.

F - forward, R - reverse

Supplementary Table S3. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C.

Accession	Locus tag/Gene	Description[a]	Regulation Ye9[b]																			
			standard conditions				26°C				37°C				26°C				37°C			
			26°C vs 37°C				st.cond. vs high osm.				st. cond. vs high osm.				st. cond. vs low pH				st. cond. vs low pH			
			q-value[c]	ratio[d]	fc[e]	pep[f]	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep
Porin activity GO:0015288																						
ADZ43215	YE105_C2719	OmpC porin	0.00078	0.19	5.2	24	0.00006	0.24	4.09	22	0.8581	0.93	1.08	24	0.00005	0.15	6.58	24	0.15879	0.7	1.43	26
ADZ43059	YE105_C2563	OmpF porin	0.00169	6.91	6.91	15	1	1.47	1.47	15	0.99881	1.29	1.29	11	0.59447	1.32	1.32	15	0.75037	0.79	1.27	14
ADZ42354	YE105_C1858	PhoE porin	0.00555	9.52	9.52	11	1	0.9	1.11	9	1	0.98	1.02	18	0.9462	1.11	1.11	11	0.92753	0.84	1.19	19
ADZ41063	YE105_C0565	Sucrose porin ScrY	0.00159	9.71	9.71	21	0.00766	1.74	1.74	23	1	0.97	1.03	12	0.00005	3.34	3.34	22	0.71623	0.9	1.11	11
ADZ44076	YE105_C3582	Maltoporin LamB	0.00003	18.8	18.8	24	0.652	1.13	1.13	24	1	1	1	22	0.40518	1.31	1.31	24	0.41628	0.71	1.4	20
ADZ40635	YE105_C0137	Vitamin B12 transporter BtuB	0.56359	1.49	1.49	20	0.9115	0.76	1.31	19	0.41461	0.51	1.96	18	1	0.93	1.07	22	0.02069	0.54	1.85	22
Transporter activity GO:0005215																						
ADZ43857	YE105_C3363	Type I secretion OM protein TolC	0.00263	2.4	2.4	50	1	1.08	1.08	48	0.83826	1.31	1.31	50	1	1.1	1.1	48	1	0.96	1.04	50
ADZ40597	YE105_C0099	Multidrug resistance protein D	0.86486	0.58	1.72	4					0.91931	1.23	1.23	4	0.02181	0.2	5.07	4	0.01314	0.36	2.78	4
ADZ44049	YE105_C3555	Putative Na(+)/H(+) exchanger protein	0.86789	0.9	1.11	7	0.82336	1.79	1.79	5	0.84284	1.23	1.23	8	0.02667	2.95	2.95	5	0.51777	1.65	1.65	7
ADZ42774	YE105_C2278	Periplasmic oligopeptide-binding protein OppA	0.0152	5.91	5.91	21	0.001	1.95	1.95	21	0.96443	0.72	1.38	12	0.64866	1.3	1.3	21	0.85432	0.59	1.69	11
ADZ40696	YE105_C0198	ABC transporter permease	0.00051	0.27	3.69	34	1	1.4	1.4	27	0.0108	2.22	2.22	38	0.00332	0.57	1.76	32	0.00274	1.77	1.77	37
ADZ41495	YE105_C0999	DL-methionine transporter substrate-binding subunit	0.00125	0.25	4.08	26	1	1.09	1.09	23	0.90377	0.99	1.01	26	1	1.01	1.01	22	0.94001	0.98	1.02	26
ADZ41961	YE105_C1465	D-galactose-binding periplasmic protein MglB	0.0567	3.21	3.21	14	0.00299	2.98	2.98	14	1	0.58	1.73	11	0.462	1.52	1.52	14	0.78387	0.68	1.47	11
ADZ44078	YE105_C3584	Maltose ABC transporter periplasmic protein MalE	0.54298	5.2	5.2	17	0.00006	3.37	3.37	17	1	0.39	2.59	4	0.42697	1.66	1.66	17				
ADZ41803	YE105_C1307	Translocation protein TolB	0.90598	1.14	1.14	28	0.00006	2.52	2.52	28	0.93261	1.3	1.3	28	1	0.94	1.06	28	0.31981	1.4	1.4	27
ADZ41295	YE105_C0799	Protein translocase subunit SecA	0.04197	0.38	2.61	33	1	0.88	1.14	29	0.76605	1.26	1.26	31	1	0.95	1.05	29	0.33329	1.25	1.25	31
Gram-negative-bacterium-type cell OM assembly GO:0043165																						
ADZ43507	YE105_C3013	BamB	0.1853	2.28	2.28	20	0.00059	1.87	1.87	20	0.98771	0.97	1.03	15	0.39985	1.22	1.22	20	1	1.02	1.02	16
ADZ43450	YE105_C2956	BamC	0.01099	1.93	1.93	37	0.16485	1.39	1.39	37	0.79659	1.34	1.34	34	0.00047	1.31	1.31	38	0.83625	1.13	1.13	35
ADZ43584	YE105_C3090	BamE	0.85797	1.04	1.04	7	0.29072	1.55	1.55	7	0.92118	1.06	1.06	7	0.01789	1.85	1.85	7	0.75062	0.93	1.08	7
ADZ41474	YE105_C0978	BamA	0.00003	2.53	2.53	96	1	1.05	1.05	97	0.02721	1.37	1.37	80	0.77749	1.11	1.11	98	1	1	1	83
ADZ41154	YE105_C0656	BamD	0.08787	2.18	2.18	25	0.00006	2.27	2.27	23	0.83647	1.68	1.68	21	0.00104	1.48	1.48	24	0.52102	1.33	1.33	24
ADZ41135	YE105_C0637	LPS-assembly protein LptD	0.01326	1.97	1.97	73	1	1.11	1.11	75	0.41393	1.44	1.44	65	1	1.16	1.16	74	0.41224	0.83	1.21	67
ADZ41475	YE105_C0979	Chaperone protein Skp	0.25032	6.86	6.86	4	0.02631	3.78	3.78	4	1	0.91	1.09	2	1	1.27	1.27	6	0.73648	0.73	1.38	3

Accession	Locus tag/Gene	Description[a]	Regulation Ye9[b]																			
			standard conditions				26°C				37°C				26°C				37°C			
			26°C vs 37°C				st.cond. vs high osm.				st. cond. vs high osm.				st. cond. vs low pH				st. cond. vs low pH			
			q -value[c]	ratio[d]	fc[e]	pep[f]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
Biosynthetic process GO:0009058																						
ADZ42901	YE105_C2405	Lipid A palmitoyltransferase PagP					0.48641	1.83	1.83	6					0.01703	2.13	2.13	6				
ADZ42005	YE105_C1509	WbcU protein	0.0243	3.8	3.8	23	1	0.79	1.26	25	1	0.98	1.02	14	1	1.02	1.02	25	0.73453	1.21	1.21	11
Iron ion homeostasis GO:0055072																						
ADZ40857	YE105_C0359	Hemin receptor HemR	0.86768	1.12	1.12	22	1	1.02	1.02	25	1	0.88	1.13	20	1	0.86	1.17	23	0.02781	0.59	1.7	20
ADZ42893	YE105_C2397	Ferrichrome receptor protein FcuA	0.00017	2.84	2.84	60	1	1.08	1.08	62	0.93127	0.76	1.31	54	1	0.91	1.1	61	0.00005	0.52	1.94	58
ADZ41093	YE105_C0595	Heme ABC exporter, ATP-binding protein CcmA	0.11849	0.11	9.16	34					0.4886	1.53	1.53	34	1	1.01	1.01	15	0.00016	1.69	1.69	33
ADZ44135	YE105_C3641	Bacterioferritin Bfr	0.33112	2.23	2.23	13	0.08105	0.51	1.96	13	1	0.85	1.17	8	0.03035	2.21	2.21	13	0.01904	0.49	2.03	9
Pathogenesis GO:0009405																						
ADZ44497	YE105_P0064	Adhesin YadA	0.00003	0.07	15.34	44	1	1.04	1.04	24	0.83129	0.9	1.11	47	1	1	1	23	0.40197	0.83	1.2	46
EOR65641	YE149_21381	T3SS effector protein YopE	0.00003	0.02	59.37	23	1	1.23	1.23	16	0.13809	1.6	1.6	23	0.727	1.29	1.29	15	0.00623	1.69	1.69	23
ADZ44479	YE105_P0046	Tyrosine phosphatase Yop effector YopH	0.00003	0.02	62.61	59	0.83537	1.48	1.48	16	0.45355	1.29	1.29	62	0.80618	1.06	1.06	13	0.00035	1.58	1.58	60
ADZ44467	YE105_P0034	YscC secretin	0.00015	0.13	7.6	29	1	1.29	1.29	16	0.80615	0.72	1.39	28	1	1.06	1.06	17	0.11431	0.75	1.33	28
ADZ40701	YE105_C0203	Phospholipase A YplA	0.82175	1.24	1.24	20	0.00006	0.46	2.19	20	1	0.98	1.02	18	0.96642	0.82	1.23	20	0.73563	0.77	1.3	18
ADZ42189	YE105_C1693	Invasin Inv	0.00003	4.6	4.6	23	1	0.84	1.19	24	0.80615	1.05	1.05	21	0.03246	1.39	1.39	25	0.95095	0.92	1.08	22
ADZ43157	YE105_C2661	OM usher protein MyfC																	0.00992	0.09	11.33	12
ADZ43625	YE105_C3131	Urease subunit gamma UreA	0.00003	17.15	17.15	25	1	1.19	1.19	25	0.02412	0.55	1.82	14	1	1.07	1.07	26	0.51278	0.52	1.92	16
ADZ43624	YE105_C3130	Urease subunit beta UreB	0.00077	44.14	44.14	8	0.34823	2.17	2.17	9	0.80841	0.43	2.3	5	0.61054	1.76	1.76	9	0.41732	0.55	1.82	5
ADZ43623	YE105_C3129	Urease subunit alpha UreC	0.00003	18.64	18.64	70	1	1.26	1.26	71	0.00029	0.5	2	45	1	0.99	1.01	72	0.00005	0.36	2.82	48
ADZ43620	YE105_C3126	Urease accessory protein UreG	0.47973	0.64	1.56	19	0.87646	0.69	1.46	18	0.80841	1.28	1.28	19	0.00994	0.56	1.79	17	0.80262	1.12	1.12	21
Response to stress GO:0006950																						
ADZ43616	YE105_C3122	Acid stress chaperone HdeB	0.14523	4.65	4.65	5	0.04414	2.27	2.27	5	0.57479	0.58	1.71	7	0.86758	1.32	1.32	6	0.73834	0.77	1.3	8
ADZ42722	YE105_C2226	Carbon starvation protein A CstA	0.00003	5.49	5.49	29	1	1.16	1.16	29	0.54315	0.81	1.24	26	0.00466	1.46	1.46	29	0.95095	1	1	24
ADZ42566	YE105_C2070	Phage shock protein PspA	0.14865	0.44	2.3	13	1	0.91	1.1	12	0.39176	0.68	1.46	13	1	1.08	1.08	12	0.00005	0.28	3.54	13
ADZ41933	YE105_C1437	DNA protection during starvation protein	0.93902	0.46	2.18	12	1	0.69	1.46	8	1	1.02	1.02	12	0.01022	0.38	2.65	10	0.9592	0.94	1.07	11
ADZ41114	YE105_C0616	Chaperone protein DnaJ	0.24071	0.17	5.87	11	0.72651	0.52	1.93	4	0.05355	0.56	1.79	13	0.25174	0.4	2.51	8	0.00005	0.48	2.09	13
ADZ41113	YE105_C0615	Chaperone protein DnaK Hsp70	0.00003	0.13	7.98	56	1	1.21	1.21	30	0.04984	1.35	1.35	58	1	1.09	1.09	32	1	1.07	1.07	56
ADZ43567	YE105_C3073	Elongation factor 4 LepA	0.01227	0.18	5.52	14	0.71083	1.59	1.59	11	0.793	1.17	1.17	14	1	1.08	1.08	11	0.68513	1.23	1.23	14
ADZ40752	YE105_C0254	Carbon starvation protein	0.09871	4.39	4.39	9	0.83718	2.61	2.61	9	1	1.52	1.52	8	0.00005	12.03	12.03	9	0.08508	3.18	3.18	7
Catalytic activity GO:0003824																						
ADZ43177	YE105_C2681	Inner membrane protein YeiU	0.00375	6.99	6.99	2	1	0.81	1.24	2	0.60746	0.66	1.52	2	0.28353	0.91	1.1	2	0.58054	0.1	10.22	2

Accession	Locus tag/Gene	Description[a]	Regulation Ye9[b]																			
			standard conditions				26°C				37°C				26°C				37°C			
			26°C vs 37°C				st.cond. vs high osm.				st. cond. vs high osm.				st. cond. vs low pH				st. cond. vs low pH			
			q -value[c]	ratio[d]	fc[e]	pep[f]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ43947	YE105_C3453	Serine endoprotease	0.57635	1.77	1.77	16	1	0.91	1.1	15	0.12081	0.63	1.59	14	0.00345	1.95	1.95	15	0.8858	1.1	1.1	14
Cell motility GO:0048870																						
ADZ42196	YE105_C1700	Flagellar hook protein FlgE	0.00003	53.43	53.43	33	0.89816	0.84	1.19	32	0.10332	0.54	1.86	20	1	0.93	1.08	32	0.00019	0.36	2.75	24
Cell redox homeostasis GO:0045454																						
ADZ41184	YE105_C0688	Anaerobic dimethyl sulfoxide reductase chain A	0.00003	15.13	15.13	43	0.5212	0.82	1.23	47	0.66525	0.47	2.13	7	0.09012	1.18	1.18	48	0.59416	0.64	1.56	8
ADZ41183	YE105_C0687	Anaerobic dimethyl sulfoxide reductase chain B	0.11967	28.19	28.19	10	0.03676	0.6	1.66	11					1	0.91	1.1	10				
ADZ41796	YE105_C1300	Cytochrome D ubiquinol oxidase subunit I	0.16514	2.24	2.24	31	0.03622	0.65	1.55	32	0.58107	0.82	1.22	30	0.03768	0.74	1.35	31	1	1.02	1.02	29
Cell wall organization GO:0071555																						
ADZ43927	YE105_C3433	Penicillin-binding protein activator LpoA	0.01873	2.08	2.08	36	0.00006	1.93	1.93	36	1	1.14	1.14	33	1	1.04	1.04	38	1	0.99	1.01	33
ADZ42453	YE105_C1957	N-acetylmuramoyl-L-alanine amidase	0.40021	1.82	1.82	19	1	1.24	1.24	36	0.84226	0.94	1.06	19	0.02038	1.56	1.56	18	0.00278	0.57	1.75	19
ADZ43064	YE105_C2568	Murein L,D-transpeptidase	0.16458	3.7	3.7	14	0.83424	1.14	1.14	16	0.72213	1.28	1.28	7	0.01576	1.58	1.58	15	0.91908	1.2	1.2	8
ADZ42523	YE105_C2027	Murein L,D-transpeptidase	0.99301	0.98	1.02	18	0.02649	2.18	2.18	18	0.91943	1.32	1.32	17	0.06591	1.59	1.59	18	0.18483	1.39	1.39	17
ADZ43369	YE105_C2873	Murein hydrolase B	0.11826	5.25	5.25	9	0.20994	1.74	1.74	9	0.61949	0.52	1.92	6	0.03118	1.99	1.99	9	0.90729	1.18	1.18	6
Cell division GO:0051301																						
ADZ41291	YE105_C0795	Cell division protein FtsZ	0.04848	0.19	5.37	16	1	0.89	1.13	11	0.53128	1.46	1.46	16	0.97394	0.99	1.01	13	0.21236	1.46	1.46	16
ADZ41277	YE105_C0781	Cell division protein MraZ									0.6092	0.34	2.95	4					0.01624	0.2	5.01	4
ADZ44021	YE105_C3527	Rod shape-determining protein MreB	0.01691	0.25	3.96	18	1	1.41	1.41	18	0.82943	1.56	1.56	17	0.99861	0.99	1.01	18	0.3687	1.39	1.39	16
ADZ42415	YE105_C1919	Septum site-determining protein MinD	0.0274	0.3	3.34	16	1	1.07	1.07	16	0.58554	1.34	1.34	16	1	0.93	1.07	15	0.19537	1.54	1.54	16
Undefined GO term																						
ADZ41640	YE105_C1144	Lipoprotein YscW Superfamily	0.73859	1.4	1.4	8	0.03772	1.65	1.65	8	1	1.23	1.23	8	0.001	2.11	2.11	8	0.95254	1	1	8
ADZ43548	YE105_C3054	Putative lipoprotein YfhG	0.95582	0.94	1.07	13	0.00546	1.96	1.96	12	0.06889	1.79	1.79	11	0.58485	1.26	1.26	12	1	1.04	1.04	11
ADZ43231	YE105_C2735	OM protein YfaZ	0.00264	3.97	3.97	12	1	1.11	1.11	12	1	1.94	1.94	11	0.30472	1.42	1.42	12	1	0.73	1.36	11
ADZ43116	YE105_C2620	Putative lipoprotein	0.00093	3.05	3.05	17	0.97361	1.07	1.07	17	0.29256	1.63	1.63	16	0.59959	1.15	1.15	17	1	1	1	16
ADZ41163	YE105_C0667	Putative exported protein	0.0454	15.9	15.9	9	0.04108	1.98	1.98	9	0.79659	1.04	1.04	5	0.00424	2.42	2.42	9	0.80943	1.28	1.28	4
ADZ43544	YE105_C3050	Putative integral membrane protein	0.00053	13.17	13.17	12	1	1.1	1.1	14	0.94107	1.57	1.57	8	1	0.97	1.03	15	0.70861	1.07	1.07	8
ADZ43148	YE105_C2652	Putative OM receptor	0.00937	2.35	2.35	33	1	0.85	1.17	34	0.79751	0.74	1.35	31	1	0.86	1.16	32	0.00065	0.52	1.93	32

Accession	Locus tag/Gene	Description[a]	Regulation Ye9[b]																			
			standard conditions				26°C				37°C				26°C				37°C			
			26°C vs 37°C				st.cond. vs high osm.				st. cond. vs high osm.				st. cond. vs low pH				st. cond. vs low pH			
			q -value[c]	ratio[d]	fc[e]	pep[f]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ43106	YE105_C2610	Putative virulence factor									1	1.25	1.25	8					0.01689	0.37	2.73	8
ADZ42859	YE105_C2363	Putative envelope protein	0.10286	0.37	2.67	12	1	1.23	1.23	11	0.98058	1.08	1.08	12	0.7684	1.21	1.21	12	0.00127	1.93	1.93	12
ADZ42790	YE105_C2294	Putative exported protein	0.02472	5.7	5.7	5	0.13822	1.77	1.77	6	1	0.92	1.09	5	0.14985	1.89	1.89	6	0.91854	0.9	1.11	5
ADZ42677	YE105_C2181	Putative exported protein	0.73129	1.4	1.4	6	1	1.12	1.12	6	0.22628	1.45	1.45	6	0.00415	1.84	1.84	6	0.40134	1.24	1.24	6
ADZ41729	YE105_C1233	Rare lipoprotein A	0.42905	2.26	2.26	18	0.00159	2.09	2.09	18	1	0.95	1.05	14	0.0006	1.72	1.72	18	1	1.06	1.06	15
ADZ40924	YE105_C0426	Hypothetical protein	0.16555	1.8	1.8	29	0.00006	2.12	2.12	28	0.94107	1.26	1.26	23	0.00005	2.47	2.47	29	0.4468	1.34	1.34	25

[a]Description of the identified proteins of OMsl (outer membrane-enriched sarcosyl-insoluble fractions) according to the UniProt databases or GenBank, or of homologous sequences obtained using BLAST. Proteins were clustered based on gene ontology (biological process) terms.

[b]Proteins whose production differed due to growth under particular physicochemical conditions, according to MS analysis. St. cond. – standard conditions (LB medium); high osm. – high osmolarity (LB supplemented with NaCl to 386 mM); low pH (LB adjusted to pH 5.0), at 26°C and 37°C.

[c]q -value ≤ 0.05 – statistically significant differences in production. q -value > 0.05 are marked in red.

[d]Significant changes in protein production are defined by a ratio of abundance of ≤ 0.67 (protein more abundant at 37°C or at high osm. and low pH) or ≥ 1.5 (protein less abundant at 37°C and high osm. and low pH).

[e]fc – fold change

[f]pep – number of identified peptides belonging to the differentially produced protein

Supplementary Table S4. Comparison of the patterns of OMsl proteins produced by *Y. enterocolitica* wild-type strain Ye9 and isogenic *ompR* mutant AR4 grown under standard conditions (LB medium), high osmolarity (LB supplemented with NaCl to 386 mM) or low pH (LB adjusted to pH 5.0), at 26°C and 37°C.

Differential proteins				Regulation Ye9 vs AR4[c]																							
				standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q*-value[d]	ratio[e]	fc[f]	pep[g]	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep	*q*-value	ratio	fc	pep
Porin activity GO:0015288																											
ADZ43059	YE105_C2563	OmpF porin	OM	0.00005	11.6	11.6	15	1	1.39	1.39	11	0.00005	8.03	8.03	15	0.06074	2.43	2.43	10	0.00004	8.16	8.16	15	0.01836	3.3	3.3	14
ADZ43215	YE105_C2719	OmpC porin	OM	0.11687	2.19	2.19	17	0.00005	3.86	3.86	24	0.00005	7.39	7.39	22	0.00031	1.97	1.97	23	0.00004	10.2	10.2	24	0.00005	2.98	2.98	25
ADZ42354	YE105_C1858	PhoE porin	OM	0.00005	7.28	7.28	11	0.00939	2.08	2.08	16	0.00005	7.07	7.07	9	0.83262	3.51	3.51	16	0.00051	5.64	5.64	11	0.46399	2.96	2.96	19
ADZ41941	YE105_C1445	OmpX	OM	0.30566	3.35	3.35	3	0.75647	1.26	1.26	3	0.11888	2.99	2.99	3	0.96076	1.15	1.15	3	0.03748	5.69	5.69	3	0.3344	2.02	2.02	3
ADZ44282	YE105_C3788	Oligogalacturonate-specific porin KdgM2	OM	0.00005	0.01	120	39	0.00005	0.09	10.57	27	0.00005	0.01	183.9	39	0.00249	0.24	4.1	28	0.00004	0.01	145.3	36	0.00021	0.06	15.68	29
ADZ42758	YE105_C2262	OmpW	OM	0.97851	1.95	1.95	21	0.223	0.59	1.69	14	1	1.64	1.64	20	0.0321	0.43	2.31	13	1	0.87	1.14	21	0.00591	0.27	3.72	16
ADZ40635	YE105_C0137	Vitamin B12 transporter BtuB	OM	0.98101	0.79	1.27	22	0.33612	0.51	1.98	22	0.6672	0.62	1.61	24	0.04294	0.49	2.03	21	0.59558	0.69	1.45	25	0.14904	0.62	1.62	22
ADZ41063	YE105_C0565	Sucrose porin ScrY	OM	0.00009	2.76	2.76	22	0.00577	0.12	8.44	18	0.19228	1.44	1.44	22	0.07662	0.26	3.86	17	1	0.85	1.18	20	0.04341	0.19	5.33	17
Transporter activity GO:0005215																											
ADZ42555	YE105_C2059	Dipeptide/tripeptide permease A DtpA/TppB	IM	0.03801	15.81	15.81	5	0.05894	4.88	4.88	8	0.16247	4.41	4.41	7	0.07162	3.05	3.05	7	0.0089	16.4	16.4	6				
CBY28945	Y11_35851	Anaerobic C4-dicarboxylate transporter DcuA	IM	0.00179	10.89	10.89	5	0.21541	4.37	4.37	3	0.03178	16.39	16.39	5					0.00804	14.67	14.67	5				
ADZ44176	YE105_C3682	Nitrite transporter NirC	IM	0.12762	4.45	4.45	4	0.11089	3.99	3.99	5	0.08852	3.57	3.57	4	0.04643	6.05	6.05	5	0.13746	3.87	3.87	4	0.15114	8.79	8.79	5
ADZ42774	YE105_C2278	Periplasmic oligopeptide-binding protein OppA	P	0.00009	5.36	5.36	20	0.57854	1.34	1.34	10	0.00436	2.8	2.8	18	0.96194	1.14	1.14	11	0.0001	4.27	4.27	19	0.51147	1.32	1.32	11
ADZ42770	YE105_C2274	Oligopeptide transport ATP-binding protein OppF	IM	0.48744	1.64	1.64	17	0.83115	1.33	1.33	16	0.00675	2.98	2.98	17	0.74018	1.92	1.92	3	0.15265	3.05	3.05	17	0.1897	1.85	1.85	15
ADZ42771	YE105_C2275	Oligopeptide transporter ATP-binding protein OppD	IM	0.96006	1.18	1.18	10	0.47768	1.72	1.72	11	0.0152	2.53	2.53	11	0.26384	2.18	2.18	8	0.13241	2.72	2.72	11	1	1.69	1.69	8
ADZ44050	YE105_C3556	Putative xanthine/uracil permease	IM	0.31127	2.48	2.48	9	0.22695	2.04	2.04	9	0.01338	2.13	2.13	9	0.22575	2	2	8	0.01266	2.32	2.32	9	0.13474	1.68	1.68	8
ADZ41508	YE105_C1012	OM efflux protein	OM	0.00062	2.78	2.78	15	0.25697	0.73	1.37	7	0.01559	1.8	1.8	16	0.67107	0.91	1.09	4	0.44406	1.93	1.93	17	0.81432	1.27	1.27	9

Differential proteins				Regulation Ye9 vs AR4[c]																							
				standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q* -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ41657	YE105_C1161	Multidrug efflux protein AcrA	IM	0.30454	0.66	1.52	21	0.11173	1.62	1.62	21	0.54052	0.67	1.49	3	0.04337	1.49*	1.49	17	1	0.91	1.1	19	0.00476	2.13	2.13	17
ADZ41656	YE105_C1160	Multidrug efflux protein AcrB	IM	1	1.06	1.06	92	0.01293	1.24*	1.24	92	1	0.97	1.03	92	1	1.08	1.08	92	1	1.02	1.02	91	0.07622	1.22	1.22	91
ADZ43362	YE105_C2866	ABC transport system substrate-binding protein	P	0.00249	2.91	2.91	11	0.16682	2.23	2.23	12	0.00486	3.46	3.46	11	0.06542	2.43	2.43	8	0.00095	2.71	2.71	11	0.21099	2.26	2.26	10
ADZ44078	YE105_C3584	Maltose ABC transporter periplasmic protein MalE	P	0.00029	2.54	2.54	18	0.91505	0.85	1.18	3	0.73174	1.28	1.28	14					0.00464	2.17	2.17	17				
ADZ44153	YE105_C3659	Putative sugar transferase	IM	0.45927	1.82	1.82	5	0.02712	2.65	2.65	9	0.48502	1.49	1.49	4	0.31309	2.33	2.33	7	0.79866	1.69	1.69	6	0.45704	2.16	2.16	7
ADZ42972	YE105_C2476	Glucose-specific PTS system IIBC components	IM	0.54501	1.19	1.19	15	0.81259	0.87	1.15	14	0.01344	1.85	1.85	14	1	0.87	1.15	11	0.03749	1.83	1.83	15	0.95545	0.91	1.1	10
ADZ41295	YE105_C0799	Protein translocase subunit SecA	IM	0.3338	0.65	1.53	30	0.01873	1.66	1.66	31	1	1.18	1.18	25	0.04058	1.78	1.78	27	1	0.85	1.18	30	0.09607	1.58	1.58	26
ADZ42257	YE105_C1761	D-alanine/D-serine/glycine permease	IM	0.01373	0.03	37.02	4	0.00968	0.03	32.2	5	0.01332	0.03	33.54	4					0.00093	0.02	44.45	4				
ADZ41741	YE105_C1245	Glutamate/aspartate transport system permease	IM	1	0.3	3.35	8					0.00119	0.2	5.12	9					0.00158	0.08	12.54	8				
ADZ42241	YE105_C1745	Proline permease	IM	0.09394	0.17	6.01	4	0.03993	0.09	11.31	8	0.0561	0.17	5.89	6	0.08942	0.14	7.23	6	0.44352	0.18	5.44	4	0.06596	0.15	6.78	6
ADZ43898	YE105_C3404	Serine/threonine transporter SstT	IM	0.02459	0.1	10.49	10	0.15988	1.01	1.01	3	0.00005	0.09	10.88	11	0.12776	0.14	7.37	9					0.24056	0.1	10.45	5
ADZ40803	YE105_C0305	Cation/acetate symporter ActP	IM	0.00017	0.14	7.12	10	0.97762	0.48	2.07	9	0.0001	0.1	9.75	10					0.00038	0.13	7.57	9	0.9593	2.3	2.3	9
EHB19555	IOK_17581	Amino acid permease	IM	0.1151	0.21	4.81	8					0.17898	0.27	3.67	8					0.01266	0.11	9.39	8				
ADZ41742	YE105_C1246	Glutamate and aspartate transporter subunit	IM	0.00431	0.24	4.21	13	0.20336	0.3	3.29	9	0.00131	0.13	7.46	13	0.32015	0.24	4.14	8	0.00031	0.18	5.57	12	0.10199	0.29	3.51	9
ADZ41044	YE105_C0546	Arginine/ornithine antiporter	IM	0.0208	0.4	2.48	10	0.3031	0.63	1.59	8	0.07277	0.37	2.7	8	0.17434	0.56	1.79	5	0.00398	0.45	2.21	8				
ADZ42170	YE105_C1674	Mg(2+) transport ATPase protein B	IM	0.00062	0.12	8.3	35	0.02151	0.24	4.25	26	0.00008	0.1	9.64	32	0.32129	0.33	3.02	24	0.00861	0.13	7.55	35	0.21128	0.54	1.84	28

Differential proteins				Regulation Ye9 vs AR4[c]																							
Accession	Locus tag/ Gene	Protein description[a]	L[b]	standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
				q -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ43328	YE105_C2832	Long-chain fatty acid OM transporter FadL	OM	0.07941	0.39	2.55	5	0.04869	0.21	4.71	4	0.00881	0.26	3.89	6	0.01014	0.13	7.46	6	0.01788	0.21	4.69	6	0.04618	0.18	5.66	5
EOR82078	YEP4_07397	Putative phosphotransferase system protein	IM	0.07381	0.39	2.53	13	0.00113	0.2	5.04	12	0.77065	0.66	1.53	12	0.0321	0.27	3.74	11	0.96909	0.67	1.49	13	0.00131	0.22	4.61	12
ADZ44370	YE105_C3876	PTS system, mannitol-specific IIABC component	IM	0.16294	0.62	1.63	29	0.05613	0.63	1.6	25	0.84812	0.86	1.17	29	0.2015	0.66	1.51	24	1	1.05	1.05	29	0.04731	0.55	1.81	27
ADZ43484	YE105_C2990	PTS system, glucitol/sorbitol-specific IIBC component	IM	0.01541	0.24	4.2	12	0.03342	0.28	3.64	11	0.42379	0.61	1.64	11	0.21716	0.58	1.73	11	1	0.75	1.33	12	0.19176	0.22	4.58	6
ADZ41346	YE105_C0850	Chloride channel protein ClcA	IM	0.05363	0.45	2.23	9	0.04344	0.24	4.18	8	0.10085	0.55	1.82	9	0.49206	0.37	2.73	9	0.01789	0.49	2.06	8	0.10985	0.34	2.9	8
ADZ43615	YE105_C3121	Voltage-gated potassium channel	IM	0.05256	0.42	2.36	8	0.62061	0.59	1.69	5	0.07551	0.42	2.38	8					0.1904	0.46	2.19	7				
ADZ41961	YE105_C1465	D-galactose-binding periplasmic protein MglB	P	0.07381	2.15	2.15	14	0.03335	0.3	3.37	12	1	1.02	1.02	8	0.13076	0.4	2.51	12	0.10017	1.76	1.76	12	0.08105	0.35	2.85	12
ADZ41046	YE105_C0548	Efflux transporter RND family	IM	0.07941	0.23	4.29	13	0.62094	0.64	1.57	10	0.05153	0.53	1.88	12	0.40136	0.54	1.84	10	0.86577	0.53	1.9	12				
ADZ43857	YE105_C3363	Type I secretion OM protein TolC	OM	0.05023	0.72*	1.39	50	0.00067	0.61	1.65	50	0.13011	0.8	1.25	49	0.00693	0.62	1.61	48	0.00004	0.62	1.62	50	0.00024	0.64	1.57	49
ADZ41495	YE105_C0999	DL-methionine transporter substrate-binding subunit	P	0.28885	0.65	1.55	24	0.00078	2.38	2.38	26	0.00008	0.5	2	25	0.00007	2.86	2.86	23	1	0.94	1.06	23	0.00005	2.9	2.9	26
Gram-negative-bacterium-type cell outer membrane assembly GO:0043165																											
ADZ43450	YE105_C2956	BamC	OM	0.00005	2.72	2.72	38	0.00477	1.78	1.78	34	0.00005	2.07	2.07	37	0.37255	1.28	1.28	30	0.00004	2.44	2.44	38	0.00014	1.8	1.8	34
ADZ41154	YE105_C0656	BamD	OM	0.16415	2.33	2.33	23	0.17633	1.46	1.46	21	0.61195	1.28	1.28	21	0.50916	0.61	1.65	19	0.0001	2.01	2.01	23	1	0.86	1.16	22
ADZ41474	YE105_C0978	BamA	OM	0.00005	1.38*	1.38	98	0.7389	0.95	1.06	81	0.00005	1.35*	1.35	97	0.06014	0.74	1.35	75	0.00004	1.35*	1.35	96	0.81398	0.91	1.1	81
ADZ42323	YE105_C1827	OM lipoprotein LolB	OM	1	1.03	1.03	10	0.1072	0.39	2.56	9	0.178	0.58	1.73	10	0.2184	0.41	2.43	8	0.84436	0.88	1.14	10	0.02718	0.22	4.5	9
ADZ41135	YE105_C0637	LPS-assembly protein LptD	OM	0.61053	1.52	1.52	73	0.00269	0.65	1.54	66	0.01317	1.37*	1.37	74	0.00007	0.56	1.79	65	0.30165	1.27	1.27	73	0.01371	0.7*	1.43	69
ADZ41736	YE105_C1240	LPS-assembly lipoprotein LptE	OM	0.55013	1.33	1.33	16	0.18284	0.66	1.52	16	0.19184	1.43	1.43	15	0.04436	0.57	1.74	16	0.37592	1.43	1.43	16	0.03816	0.67	1.5	16

Differential proteins				Regulation Ye9 vs AR4[c]																					
Accession	Locus tag/ Gene	Protein description[a]	L[b]	standard conditions								high osmolarity								low pH					
				26°C				37°C				26°C				37°C				26°C				37°C			
				q -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
Biosynthetic process GO:0009058																											
ADZ42004	YE105_C1508	WbcT protein	C	0.0163	1.73	1.73	30	0.07536	1.91	1.91	27	0.0004	2.29	2.29	27	0.01019	2.74	2.74	20	0.0001	2.24	2.24	30	0.00925	2.91	2.91	20
ADZ42006	YE105_C1510	WbcV protein	C	0.85702	1.06	1.06	3	0.02348	2.57	2.57	10	1	0.98	1.02	3	0.18544	2.52	2.52	6					0.85056	0.59	1.71	8
ADZ42005	YE105_C1509	WbcU protein	C	0.05524	1.65	1.65	23	0.92268	1.62	1.62	15	0.00219	1.93	1.93	25	0.17434	2.68	2.68	14	0.00248	1.95	1.95	25	0.92993	2.68	2.68	9
Cell wall organization GO:0071555																											
ADZ42523	YE105_C2027	Murein L,D-transpeptidase	P	1	1.24	1.24	18	0.00972	2.49	2.49	18	1	1.01	1.01	15	0.05533	0.84	1.19	16	0.17668	1.62	1.62	17	0.23854	1.41	1.41	16
ADZ42453	YE105_C1957	N-acetylmuramoyl-L-alanine amidase	P	0.0248	1.82	1.82	18	0.0149	2.13	2.13	19	0.07257	1.59	1.59	18	0.06014	1.71	1.71	16	1	1.18	1.18	18	0.04287	1.81	1.81	19
EOR80052	YE150_03940	Major OM lipoprotein Lpp	OM	0.00005	1.79	1.79	19	0.3707	1.92	1.92	19	0.00654	1.47*	1.47	21	0.00107	1.98	1.98	19	0.00004	1.9	1.9	19	0.00035	2.05	2.05	21
ADZ41339	YE105_C0843	Penicillin-binding protein 1b	P	0.89034	1.08	1.08	17	1	1.16	1.16	16	0.12277	1.74	1.74	17	0.70278	1.5	1.5	15	0.16586	1.69	1.69	18	0.04467	1.93	1.93	17
ADZ41447	YE105_C0951	Membrane-bound lytic murein transglycosylase A	P	0.88659	0.95	1.05	15	0.13671	0.46	2.16	15	0.0152	0.48	2.07	15	0.19497	0.45	2.23	14	0.67359	0.72	1.38	14	0.10278	0.44	2.25	12
Pathogenesis GO:0009405																											
ADZ43157	YE105_C2661	OM usher protein MyfC	OM					0.2415	0.28	3.6	3									1	0.95	1.05	3	0.00642	6.24	6.24	12
ADZ42189	YE105_C1693	Invasin Inv	OM	0.04362	1.87	1.87	24	0.04637	1.71	1.71	21	0.05171	1.49*	1.49	25	0.79588	1.29	1.29	19	0.85877	1.16	1.16	25	0.1474	1.58	1.58	21
ADZ44444	YE105_P0011	Transmembrane effector protein YopB	OM					0.00005	0.02	48.12	41					0.00007	0.02	42.4	42					0.00005	0.01	75.73	41
ADZ44443	YE105_P0010	Translocator protein YopD	OM					0.00005	0.02	40.37	48					0.00007	0.03	37.76	48					0.00005	0.01	71.6	48
ADZ44440	YE105_P0007	T3SS effector protein YopM	OM					0.00073	0.05	20.79	23					0.00276	0.02	42.45	21	1	0.93	1.08	6	0.07599	0.25	3.95	22
ADZ44516	YE105_P0083	Protein kinase YopO	OM	0.93703	1.2	1.2	18	0.00005	0.06	16.25	64	0.9214	0.89	1.12	15	0.00007	0.1	9.64	57	1	0.82	1.22	17	0.00005	0.04	22.77	61
ADZ44518	YE105_P0085	T3SS effector protein YopP	OM					0.00048	0.1	10.37	26					0.00057	0.08	11.94	25	1	1.2	1.2	4	0.0019	0.05	19.12	25
EOR65641	YE149_21381	T3SS effector protein YopE	OM	0.76649	0.52	1.92	19	0.00005	0.17	5.77	29	0.99059	1.51	1.51	17	0.00007	0.12	8.41	27	0.00801	0.25	3.96	19	0.00005	0.07	15.03	30
ADZ44479	YE105_P0046	Tyrosine-protein phosphatase effector protein YopH	OM	0.89379	0.89	1.12	23	0.00005	0.15	6.78	76	0.85203	0.96	1.05	17	0.00007	0.12	8.49	69	0.85265	0.71	1.4	22	0.00005	0.08	13.32	72
ADZ44435	YE105_P0002	T3SS effector protein YopT	OM	0.65707	1.21	1.21	3	0.00998	0.24	4.12	10	0.92053	1.16	1.16	3	0.18349	0.24	4.08	9					0.10699	0.2	4.97	8

Differential proteins				Regulation Ye9 vs AR4[c]																					
				standard conditions								high osmolarity								low pH					
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q* -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ44434	YE105_P0001	Type III secretion modulator of injection YopK/YopQ	OM					0.00005	0.05	18.54	18					0.0003	0.05	21.11	17					0.00005	0.03	33.89	18
ADZ44454	YE105_P0021	Type III secretion OM protein YopN	OM	0.20965	3.81	3.81	3	0.00147	0.07	14.62	12	1	0.98	1.02	3	0.04588	0.07	14.4	10	1	0.96	1.04	3	0.01486	0.06	17.46	11
ADZ44467	YE105_P0034	Secretin YscC	OM	0.00005	0.15	6.63	26	0.00005	0.13	7.48	31	0.00005	0.06	16.2	26	0.00007	0.16	6.37	31	0.00004	0.1	10.1	27	0.00005	0.13	7.66	31
ADZ44451	YE105_P0018	Type III secretion protein YscX	OM					0.0194	0.08	11.89	6					0.34986	0.18	5.52	6					0.13906	0.17	5.83	6
ADZ44455	YE105_P0022	Type III secretion apparatus H^+-transporting ATPase YscN	OM					0.0128	0.35	2.87	13					0.21592	0.49	2.04	12					0.04486	0.31	3.23	12
ADZ44457	YE105_P0024	T3SS needle length determinant YscP	OM					0.02605	0.36	2.78	13					0.26892	0.45	2.23	12					0.11434	0.42	2.39	13
ADZ44497	YE105_P0064	Adhesin YadA	OM	0.00005	0.2	4.97	34	0.00005	0.1	9.83	51	0.00005	0.1	10.37	35	0.00007	0.09	10.81	51	0.00004	0.2	5.02	37	0.00005	0.1	10.27	51
ADZ40701	YE105_C0203	Phospholipase A YplA	OM	0.81721	1.14	1.14	19	0.0187	0.48	2.07	18	0.33557	1.2	1.2	19	0.00032	0.42	2.37	18	1	0.86	1.16	19	0.01586	0.49	2.06	18
ADZ43625	YE105_C3131	Urease subunit gamma UreA	C	0.93998	0.99	1.01	26	0.70703	1.94	1.94	15	0.0005	0.41	2.44	24	0.06102	3.2	3.2	12	1	1.01	1.01	26	0.03773	2.2	2.2	15
ADZ43623	YE105_C3129	Urease subunit alpha UreC	C	0.11592	0.85	1.17	74	0.0325	2.43	2.43	42	0.00005	0.49	2.03	76	0.00007	2.89	2.89	40	0.00004	0.78*	1.28	71	0.00005	5.4	5.4	45
ADZ43620	YE105_C3126	Urease accessory protein UreG	C	0.0011	0.4	2.5	19	0.0031	1.99	1.99	19	0.06832	0.58	1.72	17	0.04997	2.05	2.05	15	0.00356	0.54	1.86	20	0.04706	2.65	2.65	18
Iron ion homeostasis GO:0055072																											
ADZ43721	YE105_C3227	Ferric anguibactin-binding protein FatB	IM	0.97782	0.91	1.1	16	0.24981	2.05	2.05	15	0.18806	0.65	1.53	15	0.01661	2.31	2.31	16	0.93077	1.08	1.08	17	0.59591	2.39	2.39	15
ADZ41314	YE105_C0818	OM receptor FepA	OM	0.05373	1.68	1.68	21	0.89442	0.68	1.47	20	0.60903	1.21	1.21	23	1	1.07	1.07	15	1	1.28	1.28	22	0.35465	1.34	1.34	20
ADZ41093	YE105_C0595	Heme ABC exporter, ATP-binding protein CcmA	IM					0.01796	1.59	1.59	34					0.64575	1.56	1.56	32	0.44142	0.58	1.72	18	0.17567	1.3	1.3	30
ADZ41067	YE105_C0569	Iron transporter FecA	OM	0.02967	0.48	2.08	19	0.03816	0.39	2.56	13	0.00358	0.39	2.53	21	0.06266	0.33	3	13	0.00017	0.44	2.3	19	0.01848	0.34	2.96	14
ADZ40857	YE105_C0359	Heme receptor HemR	OM	0.75662	0.82	1.22	23	0.04201	0.5	2.02	19	0.08224	0.65	1.54	23	0.0748	0.46	2.18	20	0.87446	0.54	1.84	25	0.04091	0.58	1.72	23
ADZ44135	YE105_C3641	Bacterioferritin Bfr	C	0.07022	0.53	1.88	15	0.02251	10.28	10.28	7	0.15743	0.67	1.5	14	0.03321	8.15	8.15	9	0.00004	0.22	4.57	14	0.02714	7.17	7.17	10

Differential proteins				Regulation Ye9 vs AR4[c]																							
				standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q* -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
Response to stress GO:0006950																											
ADZ42566	YE105_C2070	Phage shock protein PspA	IM	0.97915	1.29	1.29	12	0.91296	1.22	1.22	12	0.69829	1.47	1.47	11	0.82752	1.1	1.1	13	0.87646	1.17	1.17	11	0.00675	2.36	2.36	13
ADZ41933	YE105_C1437	DNA protection during starvation protein	C	0.25397	2.41	2.41	8	0.16866	1.78	1.78	11	0.05395	2.23	2.23	8	0.13373	2.14	2.14	10	0.24307	2.02	2.02	10	0.10208	2.54	2.54	9
ADZ41491	YE105_C0995	Copper homeostasis protein CutF	OM	0.95008	1.02	1.02	14	0.30316	0.67	1.48	13	0.01723	0.49	2.03	14	0.17703	0.42	2.37	14	0.65742	0.81	1.23	12	0.02383	0.46	2.16	14
ADZ42722	YE105_C2226	Putative carbon starvation protein A CstA	IM	0.08676	0.74	1.35	30	0.00005	0.33	2.99	26	0.0001	0.52	1.94	29	0.00731	0.41	2.43	25	0.00004	0.63	1.58	29	0.00014	0.31	3.24	26
ADZ41113	YE105_C0615	Chaperone protein DnaK Hsp70	C	0.03514	0.64	1.56	40	0.00005	0.5	2.01	61	0.66728	0.76	1.32	33	0.0748	0.59	1.69	56	0.6586	0.75	1.33	39	0.00163	0.61	1.64	57
ADZ42757	YE105_C2261	Osmotically-inducible protein Y	P	0.47535	0.48	2.08	3	0.38066	1.52	1.52	5	0.01995	0.44	2.27	6	0.98786	1.1	1.1	3	0.14914	0.47	2.12	3	0.70357	0.84	1.19	3
ADZ43049	YE105_C2553	Paraquat-inducible protein B	IM	0.70713	1.36	1.36	23	0.53475	1.21	1.21	21	0.93717	1.07	1.07	22	0.98115	1.38	1.38	20	0.32155	1.39	1.39	22	0.01064	1.9	1.9	22
Catalytic activity GO:0003824																											
ADZ43177	YE105_C2681	Inner membrane protein YeiU	IM	0.00079	12.47	12.47	2	0.66169	1.29	1.29	2					0.06221	0.38	2.65	2					0.999	1.28	1.28	2
ADZ41168	YE105_C0672	Signal recognition particle protein	C	0.55144	1.36	1.36	17	0.33409	1.38	1.38	17	0.04294	1.83	1.83	17	0.83386	1.42	1.42	14	0.88687	1.28	1.28	17	0.35763	1.54	1.54	15
ADZ40865	YE105_C0367	Keto-acid formate acetyltransferase	C	0.99044	0.91	1.1	3									0.03337	1.61	1.61	41	1	0.99	1.01	3				
ADZ43088	YE105_C2592	Formate acetyltransferase 1	C	0.95016	0.74	1.35	41	0.03362	1.51	1.51	41	0.76442	1.18	1.18	38					1	0.95	1.05	43	0.41077	1.42	1.42	41
ADZ42412	YE105_C1916	Long-chain-fatty-acid--CoA ligase FadD	C	0.00005	0.09	11.73	38	0.00416	0.23	4.32	31	0.16437	0.43	2.35	32	0.22533	0.28	3.59	11	0.00004	0.19	5.15	35	0.01513	0.23	4.44	19
ADZ40899	YE105_C0401	Protein HflC	IM	0.02437	0.31	3.28	17	0.42764	0.59	1.7	16	1	0.73	1.36	15	1	1	1	15	1	1.06	1.06	17	1	0.95	1.06	12
ADZ42794	YE105_C2298	Protease 4	IM	1	0.78	1.29	28	0.01756	0.6	1.67	26	0.57273	0.83	1.2	29	0.24533	0.59	1.69	28	0.87838	0.84	1.2	29	0.08524	0.67	1.5	28
Cell motility GO:0048870																											
ADZ42196	YE105_C1700	Flagellar hook protein FlgE	OM	1	1.17	1.17	32	0.80048	1.29	1.29	19	0.1712	0.78	1.28	33	0.08481	2.37	2.37	19	1	0.8	1.25	32	0.01486	3.07	3.07	23
ADZ42168	YE105_C1672	Putative methyl-accepting chemotaxis protein	IM	0.0113	0.2	5.05	14	0.91407	0.67	1.5	3	0.78897	0.46	2.19	10	1	1.13	1.13	4	1	0.79	1.27	14	0.96527	0.49	2.02	6

Differential proteins				Regulation Ye9 vs AR4[c]																							
				standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q* -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ42180	YE105_C1684	Methyl-accepting chemotaxis protein	IM	0.03511	0.43	2.32	15	0.37119	1.45	1.45	11	0.22161	0.61	1.63	13	0.1329	0.2	4.91	9	0.85151	0.66	1.52	11	0.99411	1.39	1.39	7
ADZ42216	YE105_C1720	Flagellar M-ring protein	IM	0.82978	0.57	1.74	28					0.22162	0.69	1.44	27	0.91233	1.06	1.06	5	0.00456	0.58	1.71	27	0.848	0.74	1.35	3
Cell redox homeostasis GO:0045454																											
ADZ41797	YE105_C1301	Cytochrome D ubiquinol oxidase subunit II	IM	0.38597	2.09	2.09	10	1	0.81	1.23	7	0.15714	1.67	1.67	10	0.98525	0.96	1.04	8	0.00832	2.56	2.56	10	0.72338	0.64	1.56	8
ADZ41796	YE105_C1300	Cytochrome D ubiquinol oxidase subunit I	IM	0.05373	1.48*	1.48	31	0.41252	1.39	1.39	29	0.00005	1.83	1.83	32	0.60065	1.17	1.17	28	0.00004	2.33	2.33	30	0.92223	1.07	1.07	28
ADZ42602	YE105_C2106	NAD(P) transhydrogenase subunit alpha	IM	0.06222	1.8	1.8	29	0.3839	1.26	1.26	27	0.00108	1.77	1.77	28	0.74869	1.22	1.22	23	0.09296	1.57	1.57	28	0.78143	0.85	1.17	25
ADZ41617	YE105_C1121	Cytochrome O ubiquinol oxidase subunit II	IM	0.30546	0.75	1.34	32	0.28866	0.8	1.25	34	0.003	0.66	1.52	37	0.22489	0.6	1.67	34	0.00418	0.71*	1.4	35	0.04779	0.66	1.51	34
Cell division GO:0051301																											
ADZ43396	YE105_C2900	Cell division protein ZipA homolog	IM	0.03009	0.28	3.52	16	0.62094	0.58	1.71	14	0.02908	0.46	2.15	15	0.98761	0.83	1.2	13	1	0.85	1.17	16	0.87054	0.87	1.14	12
ADZ41291	YE105_C0795	Cell division protein FtsZ	IM	0.05427	0.53	1.9	17	0.90388	0.91	1.1	18	0.94883	0.87	1.15	12	1	1.09	1.09	17	0.55991	0.62	1.61	16	0.90674	0.98	1.02	15
Undefined GO term																											
ADZ41569	YE105_C1073	Putative exported protein	UN													0.13223	15.88	15.88	6					0.04706	14.98	14.98	7
ADZ40718	YE105_C0220	Putative membrane protein	IM	0.002	7.34	7.34	8	0.26297	1.71	1.71	7	0.0012	5.61	5.61	9	0.82286	1.51	1.51	6	0.0025	7.44	7.44	8	0.39543	1.76	1.76	7
ADZ43361	YE105_C2865	Putative exported protein	UN	0.12762	2.5	2.5	5	0.41662	1.45	1.45	6	0.14097	2.15	2.15	6	0.54041	1.61	1.61	5	0.00915	2.53	2.53	5	0.36714	1.38	1.38	6
ADZ43548	YE105_C3054	Putative lipoprotein YfhG	UN	0.44423	1.88	1.88	12	0.10398	2.01	2.01	11	0.89863	0.88	1.14	12	0.6906	1.33	1.33	11	1	1.33	1.33	12	0.01375	2.47	2.47	11
ADZ44035	YE105_C3541	OM lipoprotein PcP	OM	0.87372	1.21	1.21	11	0.93098	1.46	1.46	13	1	0.94	1.07	13	0.29168	1.79	1.79	13	1	1.09	1.09	12	0.01495	1.86	1.86	12
ADZ43116	YE105_C2620	Putative lipoprotein	UN	0.11515	1.48	1.48	17	0.86301	1.17	1.17	16	0.18602	1.35	1.35	17	1	1	1	16	0.00646	1.51	1.51	17	0.67543	1.24	1.24	15
ADZ40804	YE105_C0306	Inner membrane protein YjcH	IM	0.01357	0.1	9.68	7	0.02625	0.03	35	5	0.00885	0.09	10.62	7	0.13211	0.04	22.24	7	0.00696	0.08	12.42	6	0.03366	0.03	37.22	5
ADZ41451	YE105_C0955	Lipoprotein	UN	0.47535	0.41	2.43	2	0.97279	0.82	1.23	4	0.05166	0.23	4.43	4	0.06014	0.1	10.37	2	0.26337	0.25	4.07	2	0.34415	0.32	3.16	2
ADZ43231	YE105_C2735	OM protein YfaZ	OM	0.64417	1.62	1.62	12	0.07446	0.38	2.6	11	0.94966	1.07	1.07	11	0.00513	0.24	4.17	11	1	1.09	1.09	12	0.00866	0.26	3.89	12

Differential proteins				Regulation Ye9 vs AR4[c]																							
				standard conditions								high osmolarity								low pH							
				26°C				37°C				26°C				37°C				26°C				37°C			
Accession	Locus tag/ Gene	Protein description[a]	L[b]	*q* -value[d]	ratio[e]	fc[f]	pep[g]	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep	*q* -value	ratio	fc	pep
ADZ41832	YE105_C1336	Uncharacterized protein	UN	0.35193	0.31	3.27	13	0.34741	0.46	2.16	3	0.24139	0.4	2.53	10					0.00695	0.25	3.97	13				
ADZ41163	YE105_C0667	Putative exported protein	UN	0.30738	0.69	1.46	9	0.21195	0.38	2.6	6	0.00059	0.31	3.28	9	0.19075	0.25	3.93	6	0.00771	0.38	2.61	9	0.2234	0.24	4.24	6
ADZ42504	YE105_C2008	Lipoprotein NlpC	OM	0.1388	0.46	2.15	7	0.93527	0.94	1.07	7	0.01748	0.31	3.22	7	0.82148	0.73	1.37	7	0.08749	0.41	2.41	7	0.9719	1.16	1.16	7
ADZ41640	YE105_C1144	Lipoprotein YscW Superfamily	OM	0.69157	0.76	1.32	8	1	0.93	1.08	8	0.00129	0.41	2.43	8	0.41365	0.64	1.57	8	0.01578	0.54	1.87	8	0.0935	0.62	1.62	8
ADZ43738	YE105_C3244	Putative OM lipoprotein	OM	0.77563	0.67	1.49	15	0.35498	0.6	1.67	10	0.01528	0.48	2.1	14	0.50068	0.42	2.37	7	0.27703	0.77	1.31	15	0.10891	0.24	4.19	11
ADZ42938	YE105_C2442	Putative exported protein	UN	0.05032	1.75	1.75	24	0.1484	0.45	2.2	21	0.12237	2.13	2.13	21	0.37341	0.62	1.62	21	0.00026	2.12	2.12	23	0.0344	0.46	2.16	20

[a]Description of the identified proteins of OMsl (outer membrane-enriched sarcosyl-insoluble fractions) according to the UniProt databases or GenBank, or of homologous sequences obtained using BLAST. Proteins were clustered based on gene ontology (biological process) terms.

[b]L - Localization; OM - integral outer membrane proteins and proteins associated with outer membrane; IM - inner membrane proteins; P – periplasmic proteins; C - cytoplasmic proteins; UN - unknown localization

[c]Proteins whose production differed between the wild-type strain Ye9 and OmpR-deficient mutant AR4, grown under particular physicochemical conditions, according to MS analysis. Standard conditions (LB medium); high osmolarity (LB supplemented with NaCl to 386 mM); low pH (LB adjusted to pH 5.0), at 26°C and 37°C.

[d]*q* -value ≤ 0.05 – statistically significant differences in production. *q* -value > 0.05 are marked in red.

[e]Significant changes in protein production are defined by a ratio of abundance of ≤ 0.67 (protein more abundant in *ompR* mutant strain) or ≥ 1.5 (protein less abundant in *ompR* mutant strain) with few exceptions (indicated by an asterisk).

[f]fc – fold change

[g]pep – number of identified peptides belonging to the differentially producted protein

Appendix S1.

Detailed description of the effect of temperature, osmolarity and pH on the membrane proteome of the wild-type *Y. enterocolitica* strain Ye9 presented in Table S3.

As a first step in our differential analysis of the OMsl proteome of *Y. enterocolitica*, samples from wild-type strain Ye9 grown under different osmolarity and pH conditions at 26°C or 37°C were qualitatively and quantitatively compared (Table S3). Proteomic analysis revealed 76 differentially expressed proteins accepted for quantification (q-value ≤ 0.05, at least 2 peptides per protein) following growth of *Y. enterocolitica* under the different conditions. The greatest impact on the OM proteome was observed in response to pH (44 proteins whose abundance changed at pH 5.0) followed by temperature (39 changes) and osmolarity (26 changes), with several proteins affected by more than one physico-chemical condition.

Temperature affected eight proteins belonging to the "Pathogenesis" Gene Ontology (GO) category, particularly those encoded by the virulence plasmid pYV (Table S3). For example, the OM secretin YscC, a structural component of the *Yersinia* Ysc-Yop T3SS injectisome, exhibited an ~8-fold increase at 37°C, while the major adhesin YadA was also more abundant at the higher temperature (15-fold increase), in agreement with previous reports (Lambert de Rouvroit et al., 1992; Skurnik and Toivanen, 1992; Wattiau and Cornelis, 1994). The MS data also revealed a strong (~60-fold) increase in the T3SS effectors YopE and YopH at 37°C in the wild-type strain. The presence of these effectors in the OM fraction may indicate their surface localization, a finding compatible with recent data suggesting that Yops can be associated with the bacterial surface prior to translocation into the host cell (Akopyan et al., 2011, Dewoody et al., 2013). Among the OMsl proteins that appeared less abundant at 37°C than at 26°C were the other major OM adhesin invasin (Inv) and the flagellar hook protein FlgE (Table S3), in accordance with previous reports (Pepe et al., 1994; Brzostek et al., 2007; Minnich and Rohde, 2007).

To examine altered protein production caused by changes in osmolarity, we examined the OMsl proteome of Ye9 cells cultured in LB medium (standard conditions) and in LB supplemented with NaCl (386 mM), at both 26°C and 37°C (Table S3). This analysis revealed 26 proteins exhibiting osmolarity-responsive changes. Interestingly, considerably more differences were detected at 26°C (20 proteins) than at 37°C (4 proteins), with no overlap between the two groups. The upregulated group consisted of only 5 proteins and included porin OmpC, in agreement with previous reports for *Y. enterocolitica* (Brzostek et al., 1989) and also *E. coli* (Russo and Silhavy, 1990). The proteins showing reduced abundance upon growth in NaCl-supplemented LB were transporters, lipoproteins, proteins involved in OM assembly or cell wall organization, chaperones and the sucrose porin ScrY (Table S3).

Finally, 44 proteins showed altered abundance at pH 5.0 compared with pH 7.0. The number of pH-responsive proteins was found to be almost equal in cells cultured at 26°C and 37°C, i.e. 25 and 19, respectively (Table S3). The proteins upregulated at pH 5.0 included porin OmpC, and urease components UreA and UreG, confirming previous reports (Heyde and Portalier, 1987; Sato et al., 2000; Hu et al., 2009a). The OM usher protein MyfC (involved in *Y. enterocolitica* Myf fimbrial assembly) was also upregulated at pH 5.0, but only at 37°C, in accordance with the known requirements for the production of the pilin MyfA, i.e. host body temperature and acid pH (Iriarte and Cornelis, 1995). Our analysis also identified the increased production of OM receptors involved in iron acquisition (FcuA for ferrichrome and HemR for heme) and proteins involved in stress responses (e.g. PspA). The group downregulated at pH 5.0 included proteins involved in cell wall organization (3 murein modification enzymes), outer membrane assembly (BamE) and LPS modifications (PagP), plus the T3SS effectors YopH and YopE, lipoproteins and sucrose porin ScrY.

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Publikacja oryginalna 2

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The Role of OmpR in the Expression of Genes of the KdgR Regulon Involved in the Uptake and Depolymerization of Oligogalacturonides in *Yersinia enterocolitica*

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Oligogalacturonide (OGA)-specific porins of the KdgM family have previously been identified and characterized in enterobacterial plant pathogens. We found that deletion of the gene encoding response regulator OmpR causes the porin KdgM2 to become one of the most abundant proteins in the outer membrane of the human enteropathogen *Yersinia enterocolitica*. Reporter gene fusion and real-time PCR analysis confirmed that the expression of *kdgM2* is repressed by OmpR. We also found that *kdgM2* expression is subject to negative regulation by KdgR, a specific repressor of genes involved in the uptake and metabolism of pectin derivatives in plant pathogens. The additive effect of *kdgR* and *ompR* mutations suggested that KdgR and OmpR regulate *kdgM2* expression independently. We confirmed that *kdgM2* occurs in an operon with the *pelP* gene, encoding the periplasmic pectate lyase PelP. A pectinolytic assay showed strong upregulation of PelP production/activity in a *Y. enterocolitica* strain lacking OmpR and KdgR, which corroborates the repression exerted by these regulators on *kdgM2*. In addition, our data showed that OmpR is responsible for up regulation of the *kdgM1* gene encoding the second specific oligogalacturonide porin KdgM1. This indicates the involvement of OmpR in the reciprocal regulation of both KdgM1 and KdgM2. Moreover, we demonstrated the negative impact of OmpR on *kdgR* transcription, which might positively affect the expression of genes of the KdgR regulon. Binding of OmpR to the promoter regions of the *kdgM2-pelP-sghX* operon, and *kdgM1* and *kdgR* genes was confirmed using the electrophoretic mobility shift assay, suggesting that OmpR can directly regulate their transcription. We also found that the overexpression of porin KdgM2 increases outer membrane permeability. Thus, OmpR-mediated regulation of the KdgM porins may contribute to the fitness of *Y. enterocolitica* in particular local environments.

Keywords: OmpR, KdgR, KdgM porins, *Yersinia enterocolitica*, pectate lyase

INTRODUCTION

Yersinia enterocolitica, an enteropathogenic bacterium of the genus *Yersinia* in the Enterobacteariaceae family is found in various ecological niches associated with the human body and free-living in the environment (Bottone, 1997). To reside in these greatly different habitats, *Y. enterocolitica* requires the ability to rapidly adapt to fluctuations in various physico-chemical factors (Straley and Perry, 1995). Adaptation to new growth conditions involves the reorganization of gene expression, which is mediated partly by two-component regulatory systems (TCSs) (Stock et al., 1989; Hoch and Silhavy, 1995). TCSs play a role in diverse signaling processes and are widespread in bacteria, including the genus *Yersinia* (Marceau, 2005; Flamez et al., 2008; O'Loughlin et al., 2010). The EnvZ/OmpR regulatory system of non-pathogenic *Escherichia coli* K-12 is the most well characterized TCS, consisting of the transmembrane histidine kinase EnvZ and response regulator OmpR, which acts to modulate gene transcription (Kenney, 2002).

Much of our knowledge of the regulatory activities of EnvZ/OmpR has been derived from studies on the regulation of the outer membrane (OM) OmpC and OmpF porins in response to changes in the osmolarity of the environment (Russo and Silhavy, 1991). OmpC and OmpF are general porins that facilitate the passive and non-specific diffusion of low molecular weight hydrophilic substances across the OM (Nikaido, 2003). OmpR is a transcription factor that plays a role in both the positive and negative regulation of the *ompC* and *ompF* genes. Besides these general porin genes, other targets of OmpR have been identified in *E. coli* and other enterobacteria. OmpR is involved in the regulation of flagella synthesis (Shin and Park, 1995), fatty acid transport (Higashitani et al., 1993), the stationary-phase acid tolerance reponse (Bang et al., 2000) and curli fiber formation (Jubelin et al., 2005). Moreover, a role for OmpR in controlling the virulence properties of pathogenic bacteria has been demonstrated (Bernardini et al., 1990; Lee et al., 2000; Hu et al., 2009; Cameron and Dorman, 2012). The function of OmpR is important in the physiology and virulence of *Y. enterocolitica* (Dorrell et al., 1998; Brzostek et al., 2003; Raczkowska et al., 2011; Skorek et al., 2013).

Outer membrane proteins (OMPs) play a crucial role in the adaptation of bacterial cells to changes in the environment (Nikaido, 2003). Changes in the OMP composition allow bacteria to adapt to diverse environments, are associated with drug resistance, and are involved in bacterial pathogenesis. Recently, a proteomic approach was used to investigate OmpR-dependent OMP expression in *Y. enterocolitica* (Nieckarz et al., 2016). Comparative LC-MS/MS analysis identified a large panel of proteins whose expression is negatively or positively regulated by OmpR. Among the OMPs that are negatively affected by OmpR, one displayed high similarity to the KdgM proteins of *Dickeya dadantii* (formerly *Erwinia chrysanthemi*), a phytopathogenic member of the Enterobacteriaceae (Hugouvieux-Cotte-Pattat et al., 1996; Blot et al., 2002; Condemine and Ghazi, 2007). Two proteins of the *D. dadantii* KdgM family, namely KdgM and KdgN, are well characterized OM porins involved in the import of long oligogalacturonides (OGAs), the products of pectin degradation (Blot et al., 2002; Condemine and Ghazi, 2007). The synthesis of these specific porins is strongly induced by the presence of pectic derivatives and is controlled by KdgR, a general repressor of operons/genes involved in pectin catabolism in *D. dadantii* (Nasser et al., 1992; Rodionov et al., 2004). The KdgR regulon of *D. dadantii* includes genes encoding secreted pectinases as well as periplasmic and cytoplasmic enzymes that participate in the depolymerization of pectin derivatives. The end product of the pectin degradation pathway is 2-keto-3-deoxygluconate (KDG), which is used as a source of carbon and energy, and as a direct effector of KdgR activity. The interaction of KdgR with KDG releases this transcriptional regulator from operators located in the regulatory regions of target genes, thus derepressing their expression (Reverchon et al., 1991; Nasser et al., 1994).

Comparative genomic analysis revealed the presence of an incomplete pectin degradation pathway in a variety of enterobacteria, including pathogenic *Yersiniae*, i.e., enteropathogenic *Y. enterocolitica* and the plague bacillus *Y. pestis* (Rodionov et al., 2004). Pathogenic *Yersiniae* lack extracellular pectinases as well as the Out transport system necessary for their secretion. Furthermore, other enzymes that are responsible for the degradation of pectin derivatives are missing. In comparison with the large number of such enzymes in *D. dadantii*, only three pectate lyases have been detected in *Y. enterocolitica* (Abbott and Boraston, 2008; Hugouvieux-Cotte-Pattat et al., 2012).

In this study we have used available genomic data and bioinformatics tools to identify an incomplete pectin degradation pathway in *Y. enterocolitica* subsp. *palearctica*, which includes two porins of the KdgM family (KdgM1 and KdgM2). The role of *Y. enterocolitica* OmpR in the modulation of *kdgM* genes expression was characterized and this regulatory activity was correlated with variations in selected physiological properties of this enteropathogen that may be important for stress resistance.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The bacterial strains used in this study are described in **Table S1**. Unless indicated, *Y. enterocolitica* strains were cultured at 26°C in LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) or in Minimal medium A (MMA; Miller, 1992). *E. coli* strains were grown at 37°C in LB medium. *Rhizobium etli* CE3 was grown at 26°C in TY medium (Beringer, 1974). As required, media were supplemented with glycerol (Gl; 0.2% w/v), polygalacturonic acid (PGA; Sigma-Aldrich) that had been hydrolyzed by autoclaving to produce a mixture of oligogalacturonides (OGAs; 0.4% w/v) (Goubet et al., 2003) or pectin (from citrus peel, 0.2% w/v; Sigma-Aldrich). L-(+)-arabinose was added to growth medium at a concentration of 0.2% (w/v) to induce expression from the arabinose-regulated promoter in pBAD18Km. Antibiotics were used for selection at the following concentrations: nalidixic acid (Nal)−30 μg/ml, chloramphenicol (Cm)−25 μg/ml, kanamycin

(Km)−50 μg/ml, gentamicin (Gm)−40 μg/ml, tetracycline (Tet)−12.5 μg/ml, trimethoprim (Tp)−50 μg/ml. To test the effect of high osmolarity, exponential phase bacterial cultures (OD_{600} ∼ 0.4-0.5) were grown at 26°C (with shaking 150 rpm) for 2 h in Nutrient Broth medium (3 g beef extract, 5 g peptone per liter) containing 0 mM, 100 mM or 350 mM NaCl.

Molecular Biology Techniques

All DNA manipulations, including polymerase chain reaction (PCR), restriction digests, ligations and DNA electrophoresis, were performed as previously described (Sambrook and Russell, 2001). The PCR was routinely performed using Taq DNA polymerase or, when fragments were used for cloning, Phusion High-Fidelity DNA Polymerase (Thermo Scientific). Oligonucleotide primers used for PCR and sequencing were purchased from Genomed S.A. (Warsaw, Poland) and are listed in **Table S2**. Plasmids used in this study are described in **Table S1**. DNA sequencing was performed by Genomed S.A.

Isolation of Outer Membrane Proteins and SDS-PAGE Analysis

Y. enterocolitica strains were grown overnight at 26°C with shaking (150 rpm) in LB medium without or with OGAs. Outer membrane proteins isolation and SDS-PAGE analysis were performed as described in Nieckarz et al. (2016). The final protein concentrations in the samples were estimated using the RC-DC protein assay (Bio-Rad) and normalized by dilution in Laemmli buffer (Sambrook and Russell, 2001). The OM samples were mixed with sample buffer and the OMPs extracted by boiling for 10 min. Samples (50 μg of protein) were separated by electrophoresis on 10 or 12% SDS-polyacrylamide gels (SDS-PAGE) and individual polypeptide bands were visualized by Coomassie blue staining. Densitometry of stained gels was performed using Bio-Rad Image Lab software.

Construction of a *kdgM2′-′rfp* Translational Fusion Plasmid

To examine the regulation of KdgM2 expression, a translational *kdgM2* fusion to *rfp* (red fluorescent protein gene) was constructed in plasmid pBBR1MCS-5 (Gm^R). An 1,125-bp DNA fragment comprised of a sequence extending from 264 bp upstream of the *kdgM2* start codon (with *kdgM2* native ribosome binding site) plus the first 10 codons of the open reading frame (ORF) fused to an 815-bp *rfp* ORF was synthesized (GeneCust, Luxembourg). This synthetic *kdgM2′-′rfp* fragment had a BamHI site at the 5′ end and an EcoRI site at the 3′ end, and these restriction endonucleases were used to clone it using the same restriction sites in vector pBluescript II SK(+) (Agilent Technologies). Plasmid DNA of the recombinant construct was digested with BamHI and EcoRI to release a 1125-bp fragment and this was then cloned into vector pBBR1MCS-5 using the same sites to give plasmid pBKRFP. This construct was introduced into *E. coli* S17-1 λ*pir* and then transferred by conjugation into *Y. enterocolitica* strain Ye9N and strain AR4, the *ompR* deletion mutant (*ΔompR*::Km). Transconjugants of Ye9N and AR4 with pBKRFP were selected on LB agar plates containing Gm and Nal and Gm and Km, respectively. To confirm the sequence of the *kdgM2′-′rfp* fusion a PCR amplicon generated with primers LL1 and RR4 (**Table S2**) was sequenced. For experiments with *Y. enterocolitica* strains carrying the Δ*kdgR*::Gm mutation, the *kdgM2′-′rfp* fusion was also expressed from plasmid pBKRFP-Tp, a derivative of pBKRFP containing a trimethoprim (Tp) resistance cassette. To construct pBKRFP-Tp, the Tp cassette from plasmid p34E-Tp on a SalI fragment was inserted into the unique SalI site of pBKRFP. Plasmid pBKRFP-Tp was transferred to the *Y. enterocolitica kdgR* mutant (strain ES1) and the *ompRkdgR* mutant (strain AR11) by triparental mating with *E. coli* TG1 (pBKRFP-Tp) as the donor and *E. coli* DH5α/pRK2013 as the helper. Transconjugants of ES1 and AR11 with pBKRFP-Tp were selected on LB agar plates containing Nal and Tp.

Measurement of RFP Fluorescence

Two hundred microliter of culture of each strain were transferred to wells of 96-well black flat-bottomed microtitre plates with a clear base (Greiner Bio-One). Absorbance at 600 nm and RFP fluorescence (excitation 555 nm; emission 632 nm) were measured using a TECAN Infinite M200PRO microplate reader. Specific RFP fluorescence was expressed as the relative fluorescence intensity (RFU) divided by the OD_{600} after subtracting the values of a blank sample. Each culture was assayed in triplicate and the reported values are the means from three independent cultures. To test the effect of high osmolarity, bacterial cultures were grown to exponential phase in NB medium containing 350 mM NaCl. Then, 200 μl of the treated or control cell suspensions were transferred to 96-well plates and OD_{600} and RFP fluorescence were measured as before.

Construction of *kdgR* and *kdgM2* Deletion Mutants

The Δ*kdgR*::Gm and Δ*kdgM2*::Gm deletion mutants of *Y. enterocolitica* Ye9N and the *ompR* mutant AR4 were constructed by homologous recombination using suicide vector pDS132 (Philippe et al., 2004). Constructs were prepared containing overlap extension PCR products to mutate *kdgR* and *kdgM2* by insertion of a Gm^R cassette via allelic exchange at the native chromosomal loci of *Y. enterocolitica*. For each gene, three DNA fragments were PCR-amplified using primers listed in **Table S2**, with *Y. enterocolitica* chromosomal DNA (for flanking regions) or plasmid pBBR1MCS-5 Gm^R (for the Gm^R cassette) as the templates. The following primer pairs were used for the construction of *kdgR* mutants: KdgR1/KdgR2 generated fragment A-a 705-bp sequence upstream of the *kdgR* ORF; KdgR3/KdgR4 generated fragment B-an 802-bp Gm^R cassette; KdgR5/KdgR6 generated fragment C-a 684-bp sequence downstream of the *kdgR* gene. The following primer pairs were used for the construction of *kdgM2* mutants: KdgM1/KdgM2 generated fragment A-a sequence comprising 437 bp upstream of the *kdgM2* gene plus the first 250 bp of the ORF; KdgM3/KdgM4 generated fragment B-an 802-bp Gm^R cassette; KdgM5/KdgM6 generated fragment C-a sequence comprising the last 74 bp of the *kdgM2* ORF plus 623 bp downstream of this ORF.

Products A, B and C were used as the template with flanking primers KdgR1 and KdgR6 (*kdgR* mutagenesis) or KdgM1 and KdgM6 (*kdgM2* mutagenesis) to generate the final PCR products. These amplicons were purified, digested with XbaI and then individually cloned into the corresponding restriction site in suicide vector pDS132, yielding constructs pDSkdgR and pDSkdgM2, respectively. These plasmids were introduced into *E. coli* S17-1 λ*pir* by transformation, with selection on chloramphenicol and gentamicin, and then sequenced to confirm the absence of errors. Finally, pDSkdgR and pDSkdgM2 were introduced into *Y. enterocolitica* strains Ye9N and AR4 by biparental mating. Transconjugants containing single crossovers of the allelic exchange plasmid integrated into the Ye9N or AR4 genomes were selected in LB supplemented with chloramphenicol, gentamicin plus nalidixic acid (Ye9N) or kanamycin (for AR4). Integration after a single crossover was verified by PCR. To force the second recombination, the single-crossover strains were plated on LB containing gentamicin and 10% (w/v) sucrose, and incubated at room temperature for 48 h. Sucrose-resistant colonies were screened for the loss of chloramphenicol resistance (encoded by the vector). The correct allelic exchange was verified for the *kdgR* and *kdgM2* mutants by PCR using the primer pairs KdgR0/KdgR7 and KdgM0/KdgM7, respectively. Sequencing of the amplified fragments confirmed that the mutagenesis was correct. The Δ*kdgR*::Gm mutants in Ye9N and AR4 were named ES1 and AR11, respectively. The Δ*kdgM2*::Gm mutants in these strains were named MN1 and AR10, respectively.

Construction of *kdgR*::*lacZ*, *pehX::lacZ* and *pelW-togMNAB::lacZ* Transcriptional Fusion Plasmids

To obtain *kdgR*::*lacZ*, *pehX::lacZ* and *pelW-togMNAB::lacZ* transcriptional fusions, DNA fragments containing the promoters of the *kdgR* and *pehX* genes and the *pelW-togMNAB* operon were amplified from *Y. enterocolitica* chromosomal DNA by PCR using the primer pairs KdgREcoRI/KdgRKpnI, PehXEcoRI/PehXKpnI and PelWEcoRI/PelWKpnI, respectively. The amplified fragments were digested with EcoRI/KpnI and cloned into the corresponding sites of reporter vector pCM132Gm [derivative of plasmid pCM132 (Marx and Lidstrom, 2001) containing a gentamicin resistance cassette; a kind gift from Dr J. Czarnecki] upstream of a promoterless *lacZ* gene. The resulting constructs were verified by PCR using the primer pair pCM132GmSPR1/pCM132GmSPR2 (flanking the EcoRI and KpnI recognition sequences) followed by sequencing of the amplicons. The constructs pCM132Gm-*kdgR::lacZ*, pCM132Gm-*pehX::lacZ* and pCM132Gm-*pelW-togMNAB::lacZ* were introduced into *E. coli* S17-1 λ*pir* and transferred by conjugation into *Y. enterocolitica* Ye9N and the *ompR* mutant AR4, selecting transconjugants on LB plates containing Gm and Nal or Gm and Km, respectively. The presence of these constructs in these *Y. enterocolitica* strains was confirmed by plasmid isolation and PCR with the primer pair pCM132GmSPR1/pCM132GmSPR2.

Construction of *Y. enterocolitica* Reporter Strains Carrying a Chromosomal *kdgM1-lacZYA*′ Reporter Fusion

To construct a *kdgM1* promoter-*lacZYA*′ fusion, a 565-bp fragment containing the *kdgM1* regulatory region was PCR-amplified from Ye9 chromosomal DNA using primer pair KdgM1X/KdgM1S (**Table S2**). The amplicon was initially cloned into the vector pDrive (Qiagen), and then the insert released by digestion with XbaI/SmaI was subcloned into suicide plasmid pFUSE cleaved with the same enzymes to place it immediately upstream of a promoterless *lacZYA*′ operon (Baumler et al., 1996). The suicide vector construct containing the *kdgM1* fragment, verified by restriction digestion and DNA sequencing, was named pFkdgM1. This plasmid was used to transform *E. coli* S17-1 λ*pir* and then introduced into *Y. enterocolitica* Ye9N, the *ompR* mutant AR4, the *kdgR* mutant ES1 and the *ompRkdgR* mutant AR11 by biparental mating. Conjugation between the donor and recipient strains was performed on LB agar plates for 18 h at room temperature. Transconjugants were then selected on LB agar plates containing antibiotics: chloramphenicol and nalidixic acid for Ye9N, chloramphenicol and kanamycin for AR4, and chloramphenicol and gentamicin for ES1 and AR11. Single-crossover homologous recombination yielded a genomic transcriptional fusion between the *kdgM1* promoter and the promoterless *lacZYA*′ operon. The correct insertion of the suicide vector was verified by PCR using one primer located upstream of the homologous region used for recombination (LPkdgM2683) and another primer within the *lacZ* gene (lacZH991) (**Table S2**), followed by sequencing of the amplicons. Strains carrying the desired transcriptional fusions were named Ye9NK1, AR4K1, ES1K1 and AR11K1.

Construction of Plasmids pkdgR-Cm and pkdgR-Tet for Complementation

To complement the *kdgR* mutation, the *kdgR* gene was cloned in plasmid expression vectors. The gene with its native ribosome binding site (rbs) was PCR-amplified from Ye9 chromosomal DNA using primer pairs KdgRorfBamHI/KdgRorfHindIII or KdgRorfKpnI/KdgRorfSacI (**Table S2**). The BamHI/HindIII *kdgR* fragment was cloned under the control of the Plac promoter in vector pHSG575 (Takeshita et al., 1987), generating plasmid pkdgR-Cm. The KpnI/SacI *kdgR* fragment was cloned under the control of the Plac promoter in vector pBBR1MCS-3 (Kovach et al., 1995) generating plasmid pkdgR-Tet. The resulting constructs were verified by DNA sequencing. Plasmid pkdgR-Cm was used to transform the *kdgR* mutant (ES1) and *ompRkdgR* mutant (AR11), both carrying the plasmid pBKRFP-Tp (expressing a *kdgM2*′-′*rfp* fusion), by electroporation and Cm^RTp^R transformants were selected. In the same way, plasmid pkdgR-Tet was introduced into the *kdgR* mutant (ES1K1) and *ompRkdgR* mutant (AR11K1), both carrying a *kdgM1-lacZYA*′ chromosomal transcriptional fusion, and Tet^R Cm^R transformants were selected.

Construction of a Plasmid for Overexpression of KdgM2

For the overproduction of KdgM2, the *kdgM2* gene was cloned under the control of the *PBAD* promoter in vector pBAD18Km. The gene with its native rbs was PCR-amplified from Ye9 chromosomal DNA using primers ARAkdgM2SacI and ARAkdgM2SphI (**Table S2**). The SacI/SphI *kdgM2* fragment was cloned in pBAD18Km, resulting in plasmid pBAD-KdgM2, which was verified by restriction digestion and DNA sequencing. Construct pBAD-KdgM2 was introduced into the wild-type strain Ye9 by electroporation and Km^R transformants were selected. To induce KdgM2 synthesis, arabinose (0.2% w/v) was added to exponential-phase cultures in liquid medium and these were examined after an additional 1 h of growth.

RT-qPCR Analysis

Y. enterocolitica Ye9 and the *ompR* mutant strains were grown overnight in LB medium+OGAs at 26°C. Approximately 10^9 bacterial cells were then harvested from each culture and total RNA was isolated using a High Pure RNA Isolation Kit (Roche). After DNase treatment of the isolated RNA, cDNA was synthesized using a NG dART RT kit (Eurx). Real-Time PCR analysis was performed using a LightCycler 480 II (Roche Applied Science) with a SensiFAST SYBR No-ROX Kit (Bioline). Primers were designed by Amplicon sp. z o. o. and they are listed in **Table S2**. Relative quantification of gene transcription was performed using the LightCycler 480 Software 1.5.1. The data were subjected to statistical analysis using Project R (version 3.2.2.) data analysis software. The 16S rRNA gene was used as an internal reference to normalize the relative amount of target cDNA.

Electrophoretic Mobility Shift Assay (EMSA)

$OmpR\text{-}His_6$ was expressed and purified as described previously (Nieckarz et al., 2016). The *in vitro* interaction between phosphorylated OmpR (OmpR-P) and the promoters of selected genes was examined using the EMSA, essentially as described previously (Nieckarz et al., 2016). The primers listed in **Table S2** were used in PCRs with *Y. enterocolitica* genomic DNA to amplify fragments comprising the regulatory regions of the genes *kdgM1*, *kdgM2* and *kdgR*. To confirm binding specificity, a 304-bp fragment of the *Y. enterocolitica* Ye9 16S rRNA gene generated by PCR using primer pair 16SR1/16SR304 (**Table S2**) was included in all binding reactions. Ethidium bromide was used to stain DNA bands in the gels, which were visualized on a UV transilluminator.

Pectinolytic Enzyme Assay

A plate assay was used to detect pectate lyase (Pel) activity in periplasmic fluid obtained using a modified osmotic shock protocol (Neu and Heppel, 1965). Pel assay medium contained 0.8% (w/v) agarose, 1% (w/v) PGA, 1% (w/v) yeast extract, 0.38 μM $CaCl_2$ and 100 mM Tris-HCl, pH 8.0 (Lee et al., 2013). Wells were made in each plate using a cut pipette tip and the bottom of each well was sealed with molten 0.8% (w/v) agarose. Exponential phase cultures grown in LB medium at 26°C were adjusted to the same OD_{600}, then 1 ml of each was centrifuged (4,000 × *g*, 15 min, 4°C). The cell pellets were resuspended in 0.5 ml of buffer containing 20% (w/v) sucrose, 1 mM EDTA, 30 mM Tris-HCl, pH 8.0 and incubated for 10 min at room temperature with gentle shaking. After centrifugation (13,000 × *g*, 10 min, 4°C), the cell pellets were resuspended in 0.5 ml of ice-cold pure water and incubated for 10 min at 4°C with gentle shaking. After centrifugation as before, 100 μl of the supernatants containing periplasmic fluid released by osmotic shock were added to wells of the Pel enzyme assay plates. Following incubation at 26°C for 48 h, 4 M HCl was poured onto the plates and the halo areas were measured.

β-galactosidase Assays

β-galactosidase assays were performed essentially as described by Thibodeau et al. (2004), using 96-well microtiter plates (Nest Sc. Biotech.) and a Sunrise plate reader (Tecan). The β-galactosidase activity was expressed in Miller units calculated as described previously (Thibodeau et al., 2004). Each assay was performed at least in triplicate.

Semi-Quantitative Reverse Transcription RT-PCR Gene Expression Analysis

Cultures of *Y. enterocolitica* Ye9 were grown overnight in LB medium at 26°C and then total RNA was isolated from 10^7 cells using a GeneMatrix Universal RNA Purification Kit (EURx). Following treatment with RNase-free DNase I (Sigma-Aldrich), the RNA was reverse-transcribed using AMV reverse transcriptase (Sigma-Aldrich) primed with random hexamers. The cDNA was used as the template in PCRs (RNA as a negative control) with primer pairs RTkdgMpelP1/RTkdgMpelP2 or RTpelPsghX1/RTpelPsghX2 (**Table S2**), specific for the *kdgM2-pelP* mRNA or the *pelP-sghX* mRNA, respectively. The amplified fragments were resolved by electrophoresis on 2% (w/v) agarose gels and visualized by staining with ethidium bromide.

Preparation of Short OGAs by Polygalacturonase Digestion of PGA

Short oligogalacturonides (sOGAs) were obtained using the protocol of Bellincampi et al. (1993). Briefly, 1 g of unmethylated polygalacturonic acid (PGA) was solubilized in 50 ml of 50 mM sodium acetate (pH 5.0). The solution was digested for 180 min with 0.03 mU/mg of *Aspergillus niger* polygalacturonase (Sigma-Aldrich). After heat inactivation of the enzyme, the reaction mixture was diluted with 50 mM sodium acetate to a concentration of 0.5% (w/v) PGA. Next, the digested PGA was precipitated with ethanol, incubating overnight at 4°C with shaking. The pellet was recovered by centrifugation (35,000 × *g*, 0.5 h, 4°C) and dissolved in 100 μl ultrapure water. The obtained sOGAs were analyzed by non-denaturing polyacrylamide gel electrophoresis and stained with ruthenium red (0.02%, w/v), as described previously (Potiggia et al., 2015).

Detection of Reactive Oxygen Species (ROS)

Intracellular production of ROS was measured using 2′,7′-dichlorofluorescein diacetate (H2DCF-DA, Molecular Probes), essentially as described by Dong et al. (2015). Exponentially growing cultures of *Y. enterocolitica* Ye9 in LB medium and *Rhizobium etli* CE3 in TY medium (OD_{600} ∼0.3) were incubated with 10 μM H2DCF-DA for 30 min. This nonpolar compound passively diffuses into cells where it is converted to H2DCF by endogenous esterases and then rapidly oxidized to highly fluorescent DCF by intracellular peroxides. Excess dye was removed by extensive washing of the cells with fresh culture medium. The bacteria were treated with sOGAs obtained by polygalacturonase digestion of PGA (50 μg/ml), polymyxin B (25 μg/ml) or cell culture medium only (control) for 20 min. Fluorescence was measured using a TECAN Infinite M200PRO microplate reader: excitation 495 nm; emission 520 nm.

Plant Tissue Maceration Assay

The plant tissue maceration assay was performed as described by Expert and Toussaint (1985). Chicory leaves were placed in sterile Petri dishes on filter paper previously moistened with sterile water. Overnight bacterial cultures of *Y. enterocolitica* strains, *E. coli* W (ATTC 9637 strain) and *Pectobacterium carotovorum* subsp. *carotovorum* (PCM 2056 strain) grown in LB medium were diluted to an OD_{600} of 0.3. Next, 10 μl of the bacterial suspensions (about 10^8 cells) were injected into the chicory leaves cut with a sterile scalpel. The plates were closed to maintain high humidity and incubated at 26°C. Leaf tissue maceration at the sites of inoculation was scored after 2–5 days.

Antimicrobial Susceptibility Testing

The antibiotic sensitivity of *Y. enterocolitica* strains was tested using a broth micro-dilution assay. Broth microdilution was performed in sterile transparent 96-well flat-bottomed microtiter plates (Nest Sc. Biotech.). Antibiotic solutions were serially diluted 2-fold in 100 μl of Mueller-Hinton broth (MHB) in 96-well plates to produce the appropriate concentration ranges. Overnight cultures of the *Y. enterocolitica* strains were diluted to 10^5 cfu/ml and 100 μl aliquots were added to wells of the plates containing the antibiotic dilution series. The plates were then incubated with shaking (150 rpm) at 26°C for 24 h. The OD_{600} was measured using a TECAN Infinite Pro M200PRO microplate reader. The following antibiotics were assayed: ampicillin-0.2 to 400 μg/ml; cefalotin-0.98 to 500 μg/ml; cefotaxime-0.06 to 32 μg/ml; ceftazidime-0.03 to 16 μg/ml; cephaloridine-0.98 to 500 μg/ml; chloramphenicol-0.05 to 25 μg/ml; tetracycline-0.02 to 10 μg/ml. To test the effect of hydrophobic trimethoprim (400 to 0.78 μg/ml) and gentamicin (400 to 0.78 μg/ml) strains were grown in MHB at 26°C, overnight. Next, to induce KdgM2 synthesis, arabinose (0.2% w/v) was added and these cultures were incubated an additional 1 h of growth, then diluted to 10^5 cfu/ml and 100 μl aliquots were incubated with the antibiotic dilution series parallel at 26°C and 37°C for 24 h. The minimal inhibitory concentration (MIC) was the lowest concentration of the antimicrobial agent that prevented bacterial growth.

Detergent Sensitivity Assay

The MICs of detergents were determined using a liquid culture assay as previously described (Zou et al., 2011). Briefly, overnight cultures of wild-type and the mutant strains were diluted 1:1,000 in LB medium containing 2-fold serial dilutions of the applied detergent, ranging from 800 μg/ml to 1.5625 g/ml for hexadecyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS). Growth was assessed after incubation at 26°C and 37°C with shaking (150 rpm) for 18 h. To induce KdgM2 synthesis, arabinose (0.2% w/v) was added to the cultures before their incubation for 18 h. The MIC of CTAB and SDS was the lowest concentration at which growth was completely inhibited. These assays were performed two times with identical results.

1-*N*-Phenylnaphthylamine (NPN) Accumulation Assay to Examine Outer Membrane Permeability

Permeability of the *Y. enterocolotica* outer membrane was determined using the NPN uptake assay as previously described (Loh et al., 1984; Zou et al., 2011). Cultures of the test strains were grown in LB medium at 26°C or 37°C to early stationary phase. To induce KdgM2 synthesis, arabinose (0.2% w/v) was added and these cultures were incubated an additional 1 h of growth. The cells were centrifuged (8,000 × g for 1 min), and washed twice in the assay buffer (5 mM HEPES pH 7.2, 137 mM NaCl). The cells were then resuspended in the same buffer and the OD_{600} was adjusted to 1.0. 100 μl aliquots of these cell suspensions were placed in triplicate into the wells of a black 96-well fluorescence microplate (Greiner Bio-One). NPN, dissolved in acetone and then diluted in the assay buffer, was added to appropriate wells of the microplate to give a bacterial OD_{600} of 0.5 and a final NPN concentration of 10 μM. Controls containing only buffer plus NPN were included. Changes in fluorescence were then recorded using a Tecan Infinite M200PRO microplate reader: excitation 355 nm; emission 402 nm. Readings were taken every 45 s for 19.5 min. Analysis of the fluorescence values was performed using Prism 7 software (v. 7.02, GraphPad). Background fluorescence (NPN in buffer only) was subtracted from the raw values, and these results were divided by the corresponding OD_{600} values. The fluorescence of the wild-type strain at time zero was defined as 100% and all other values were normalized accordingly.

Statistical Analyses

Statistical analyses were performed using Prism 7 software (v. 7.02, GraphPad). One-way ANOVA and Tukey's multiple comparison test was used to determine statistically significant differences. In addition, the statistical significance were tested using Student's *t*-test.

RESULTS

Genomic Organization of the *kdgM1, kdgM2,* and *kdgR* Loci of *Y. enterocolitica*

Bioinformatic analysis of the *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (3/O:9 bio-serotype) genome (NCBI Reference Sequence: NC_015224.1) revealed the presence of an incomplete pectin degradation pathway compared to pectinolytic phytopathogens (**Figure 1**). The main difference is the lack of genes for secreted extracellular pectinases. However, the organization of gene clusters encoding some intracellular pectinolytic enzymes and transport systems involved in the uptake and catabolism of pectin derivatives is quite similar in the genomes of *Dickeya*, *Pectobacterium* species, *Y. enterocolitica* and in other pathogenic *Yersiniae* (Rodionov et al., 2004). The genome of *Y. enterocolitica* contains the paralogous genes *kdgM1* and *kdgM2* encoding the proteins KdgM1 and KdgM2, respectively, which are highly similar to one another (62% identity) and to the KdgM (65% for both proteins) and KdgN (57% for both proteins) oligogalacturonate-specific OM channels of *D. dadantii* (**Figure S1** and **Additional File 1**). Interestingly, the *Y. enterocolitica kdgM1* gene is situated within a cluster of genes involved in the transport of OGAs into the cytoplasm, as is *kdgM*, its homolog in the *D. dadantii* genome (Rodionov

FIGURE 1 | Comparison of the genomic organization of the *kdgM1, kdgM2,* and *kdgR* loci in *Y. enterocolitica* subsp. *palearctica* 105.5R(r) with the equivalent loci of *D. dadantii* 3937 and *P. carotovorum* subsp. *carotovorum* PC1.

et al., 2004). The *Y. enterocolitica kdgM2* gene is situated within a cluster of genes involved in OGA degradation, i.e., upstream from the gene pair *pelP-sghX*, respectively encoding pectate lyase PelP and the periplasmic polygalacturonate-binding protein SghX. The equivalent to *kdgM2* in *D. dadantii*, *kdgN*, is located in the vicinity of *ompW,* encoding a putative porin (Collao et al., 2013). The *kdgR* locus encoding repressor KdgR is similarly arranged in the genomes of *Y. enterocolitica* and the *Dickeya* and *Pectobacterium* species, being linked to downstream ORF *ogl*, which encodes an enzyme responsible for the cleavage of pectic dimers (Rodionov et al., 2004; **Figure 1**). The amino acid sequences of the KdgR regulators of *Y. enterocolitica* and *Dickeya* share 88% identity (**Figure S2**).

Identification of the Protein KdgM2 in the Outer Membrane of the *Y. enterocolitica ompR* Deletion Mutant

A comparative proteomic LC-MS/MS analysis of outer membranes prepared from wild-type *Y. enterocolitica* Ye9 (bioserotype 2/O:9) and the isogenic Δ*ompR*::Km mutant strain AR4 (Brzostek et al., 2003) revealed a number of differentially expressed proteins (Nieckarz et al., 2016). The most striking OmpR-dependent change was the strong upregulation of the protein KdgM2, a member of the KdgM family of oligogalacturonide-specific porins, initially described in *D. dadantii*. When grown in LB medium at 26°C, the *ompR* mutant strain exhibited a more than 100-fold increase in the level of this protein, indicating a major role for OmpR in the repression of KdgM2 production. To confirm this finding, SDS-PAGE analysis of OMPs isolated from *Y. enterocolitica* strains differing in their OmpR content, grown in LB medium without or with added OGAs (as an inducer) at 26°C, was performed (**Figure 2A**). Comparison of the OMP profiles revealed significant alterations in that of *ompR* mutant AR4 compared to the wild-type Ye9. Apart from the known lack of OmpC/OmpF porins (Brzostek and Raczkowska, 2007) at least three proteins showed increased abundance in the OM of the *ompR* mutant. One 48-kDa protein band and two bands at around 25 kDa were excised from the *ompR* mutant gel lane and analyzed by mass spectrometry. The 48-kDa band was identified as a homolog of *E. coli* maltoporin LamB, which is required for maltose and maltodextrin uptake (Boos and Schuman, 1998), (**Figure 2A**, band a). One of the bands at ∼25 kDa was the protein KdgM2 (**Figure 2A**, band b), while the other, migrating to a position just below KdgM2, corresponded to the MltA-interacting protein MipA (**Figure 2A**, band c) (Vollmer et al., 1999). To confirm the OmpR-dependent negative regulation of KdgM2, *kdgM2* deletion mutants were constructed in both the wild-type strain Ye9N and the *ompR* mutant AR4, generating strains MN1 and AR10, respectively. SDS-PAGE analysis (**Figure 2A**) revealed that the KdgM2 protein band was absent from the gel profile of the *ompRkdgM2* double mutant (strain AR10) and this was accompanied by the appearance of a band at about 20 kDa. LC-MS/MS analysis of this band revealed a protein highly similar to the *E. coli* porin OmpW (**Figure 2A**, band d), that may be involved in the protection of bacteria against various forms of environmental stress (Hong et al., 2006).

The KdgM2 band was not visible in the gel profile of wild-type Ye9 grown in LB medium. However, following growth of this strain in LB with added OGAs, a band appeared at the position expected for KdgM2. Thus, the presence of OGAs derepressed *kdgM2* expression. The induction of oligogalacturonide-specific porins in the presence of pectin derivatives was reported previously in *D. dadantii* (Blot et al., 2002; Condemine and Ghazi, 2007). Interestingly, under derepressed conditions (LB+OGAs), a faint band appeared at the position of KdgM2 in the gel profiles of both the strain lacking KdgM2 (strain MN1) and the *ompRkdgM2* double mutant (strain AR10). This finding suggested that the expression of other OM oligogalacturonide-specific porins might be induced in *Y. enterocolitica* by OGAs, i.e., released from KdgR repression. In the view of our data presented below, we presume that the faint band corresponds to KdgM1, the second OGA-specific porin being under KdgR repression in *Y. enterocolitica*.

Taken together, the results of this SDS-PAGE analysis suggested that OmpR acts to reduce the level of KdgM2 directly and/or indirectly.

OmpR Negatively Regulates the Expression of *kdgM2*

The OmpR-dependent regulation of *kdgM2* was examined using a *kdgM2′-′rfp* translational fusion expressed from plasmid pBKRFP. Expression of this fusion was examined by quantifying RFP fluorescence in both the wild-type and *ompR* mutant strains carrying pBKRFP, following growth to exponential and stationary phase in LB without or with added OGAs as an inducer (**Figures 2B,C**). Compared to wild-type strain Ye9N, the *ompR* mutant displayed a ∼1.3-fold increase in *kdgM2* expression when cultured in LB alone to either growth phase. When the LB was supplemented with OGAs, expression of the fusion in the wild-type strain was upregulated 2.5-fold and 2.3-fold in the exponential and stationary phases, respectively, indicating the release from the repressive activity of KdgR. In the *ompR* mutant grown in the presence of OGAs, the expression of *kdgM2* was ∼1.5-fold higher than that observed in the wild-type strain. To confirm that the lack of OmpR leads to derepression of *kdgM2*, plasmid pHR4 carrying the wild-type *ompR* allele was used to complement the *ompR* mutation in strain AR4. This caused a clearly visible reduction in the expression of *kdgM2* in LB+OGAs, indicating that OmpR negatively regulates *kdgM2*. The lack (stationary phase) or only slightly visible (exponential phase) effect of complementation observed in LB medium alone (i.e., under KdgR-repressed conditions) suggested that OmpR might influence *kdgM2* expression in different ways.

The EnvZ/OmpR regulatory system has been shown to be involved in the osmoregulation of porin expression (Pratt et al., 1996). Thus, we were curious to see if *kdgM2* expression is subject to such regulation. The expression of the *kdgM2′-′rfp* fusion was therefore tested in strains grown in NB medium+OGAs without additions or supplemented with 100 mM or 350 mM NaCl (high osmolarity) (**Figure 2D**). A decreased level of RFP

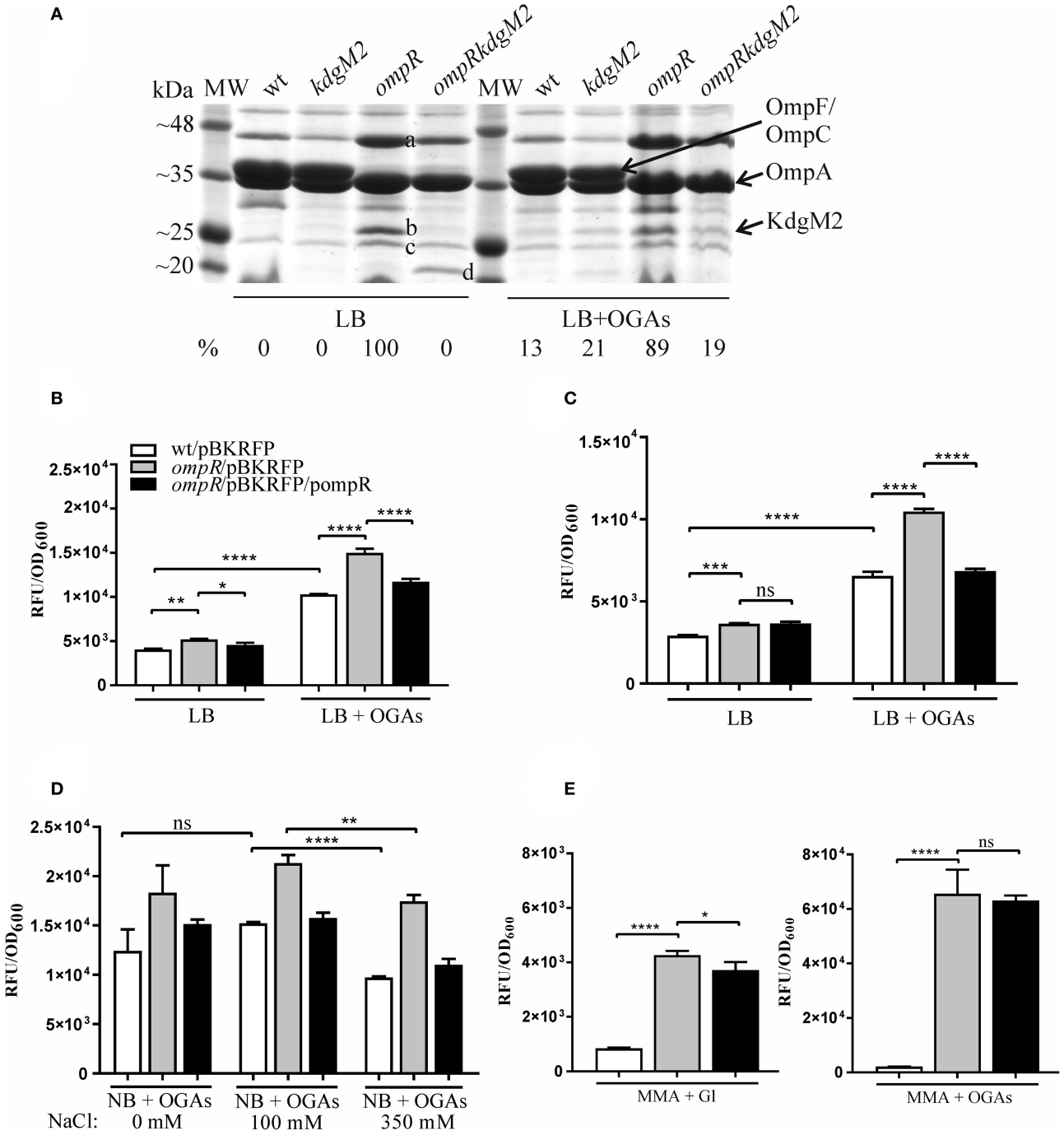

FIGURE 2 | OmpR-dependent inhibition of KdgM2 production **(A)** and *kdgM2* expression **(B–E)**. **(A)** Production of KdgM2 was analyzed in outer membrane fractions prepared from bacterial cells grown overnight in LB medium without or with added OGAs at 26°C. The Coomassie blue-stained SDS-PAGE gel shows OMPs isolated from wild-type strain Ye9, *kdgM2* mutant MN1, *ompR* mutant AR4, and *ompRkdgM2* double mutant AR10. The relative intensities of the KdgM2 bands compared to the *ompR* mutant strain in LB (which was set to 100%) are indicated. The protein bands marked a, b, c, and d were excised and identified using LC-MS/MS. MW—molecular weight standards (3-Color Prestained Protein Marker, DNA-Gdańsk). The 12% SDS-polyacrylamide gel shown is representative of the results of an experiment performed several times. **(B–E)** RFP fluorescence intensity of strains Ye9N (wild-type), AR4 (*ompR* mutant) and complemented AR4 (*ompR*/pompR) containing a *kdgM2*′-′*rfp* translational fusion expressed from plasmid pBKRFP. Strains were cultivated in LB medium without or with OGAs at 26°C to exponential **(B)** or stationary **(C)** phase, and RFP fluorescence was measured. The effect of osmolarity **(D)** was analyzed by culturing strains at 26°C to exponential phase (2 h incubation) in NB medium with OGAs (0 mM NaCl) and supplemented with 100 mM or 350 mM NaCl (high osmolarity). **(E)** Strains were cultivated in MMA medium with glycerol (Gl) or with OGAs at 26°C to exponential phase. The data represent mean fluorescence activity values normalized to the OD_{600} of the culture (± standard deviation) from two independent experiments performed in triplicate. Significance was calculated using one-way ANOVA [ns (non-significant) $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$].

fluorescence was found in the wild-type strain after exposure to high osmolarity. This effect was also observed in the *ompR* mutant, suggesting that osmoregulation of *kdgM2* is independent of OmpR and may involve other regulatory mechanisms.

Since the upregulation effect on *kdgM2* expression mediated by *ompR* deletion in the strains grown in LB medium was not as strong as anticipated (only ∼1.3-fold), we examined expression of the *kdgM2′-′rfp* fusion in strains grown to exponential phase in minimal medium (MMA) supplemented with glycerol or OGAs as the carbon source. As seen in **Figure 2E** (and later in the text, **Figure 3B**), induction of *kdgM2* expression in the wild-type strain Ye9N grown in MMA+OGAs (∼2.3-fold) was similar to that observed in LB+OGAs (∼2.5-fold). Compared to the parent strain Ye9N, the *ompR* mutant expressed 4.5-fold more *kdgM2* in MMA+glycerol and 30-fold more in MMA+OGAs. No effect of complementation of the *ompR* mutation was observed in MMA+OGAs and only slightly visible effect was noted in MMA+ Gl (i.e., under KdgR-repressed conditions). Together these results demonstrated that when grown in either LB or MMA the *ompR*-negative strain exhibited a significantly elevated level of *kdgM2* expression and KdgM2 production compared to the parental strain. This phenotype was strongest in MMA supplemented with OGAs. From these data it may be speculated that OmpR is involved in the negative regulation of *kdgM2* expression. However, the complementation analysis indicated that the link between KdgM2 and OmpR might be more complex,

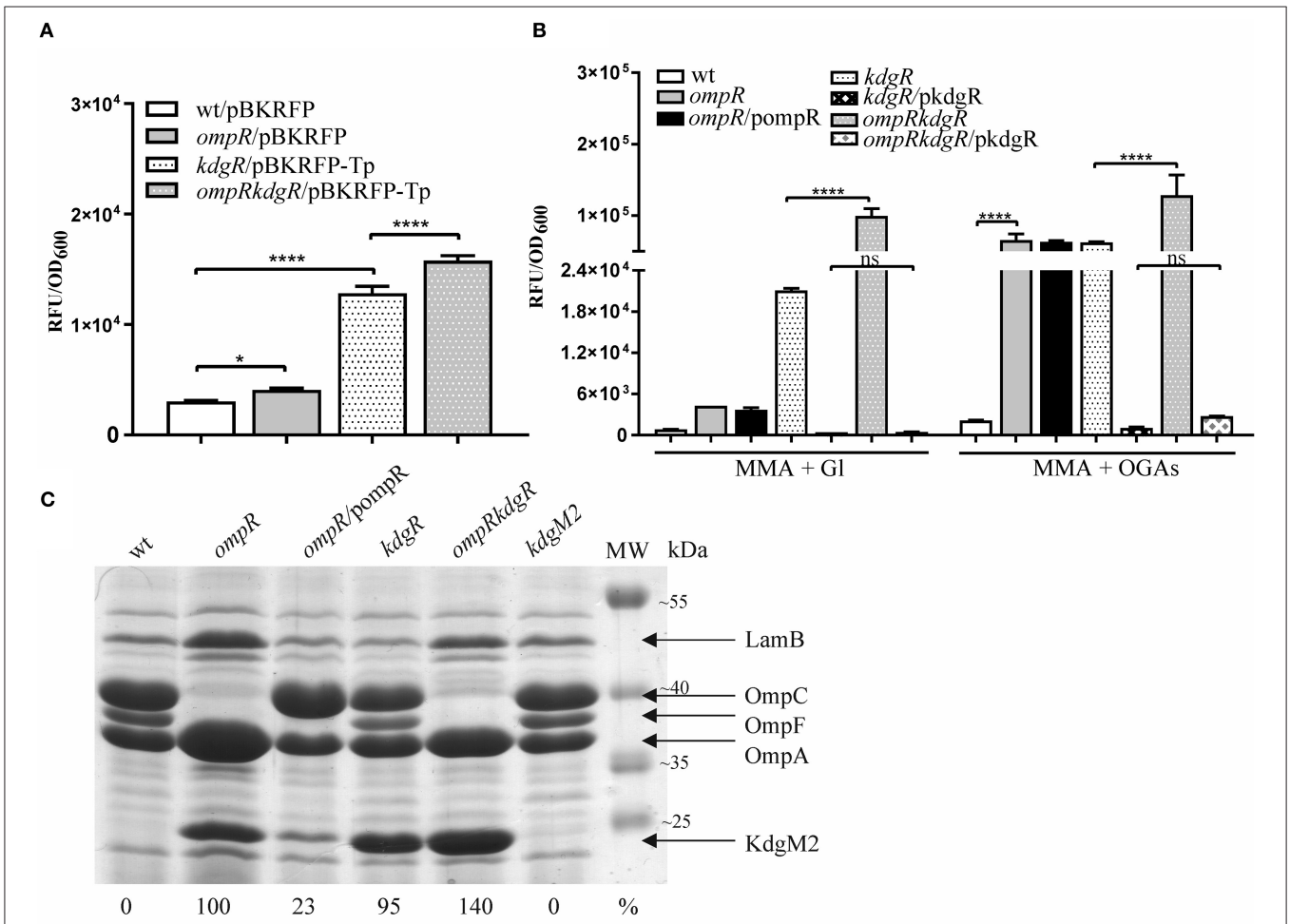

FIGURE 3 | Complex regulation of *kdgM2* expression **(A,B)** and KdgM2 porin production **(C)** by OmpR and KdgR regulators. **(A,B)** RFP fluorescence intensity of strains Ye9N (wild-type), AR4 (*ompR* mutant), ES1 (*kdgR* mutant) and AR11 (*ompRkdgR* mutant) containing a *kdgM2′-′rfp* translational fusion expressed from plasmids pBKRFP or pBKRFP-Tp. Strains were cultivated in LB medium **(A)** or MMA+Gl and MMA+OGAs **(B)** at 26°C to exponential phase. Data represent mean fluorescence activity values normalized to the OD_{600} of the culture (±standard deviation) from two independent experiments performed in triplicate. Significance was calculated using one-way ANOVA [ns (non-significant) $P > 0.05$, *$P < 0.05$, ****$P < 0.0001$]. **(C)** Absence of OmpR and KdgR independently leads to increased KdgM2 abundance. SDS-PAGE analysis was used to compare KdgM2 protein levels in the OM protein profiles of wild-type strain Ye9, the *ompR* mutant AR4, *ompR*/pompR (trans-complemented strain AR4), the *kdgR* mutant ES1, the *ompRkdgR* mutant AR11 and the *kdgM2* mutant MN1. The percentages indicate the KdgM2 band intensities in the tested strains relative to that in the *ompR* mutant AR4. The analyzed OM fractions were isolated from bacterial cells grown overnight in LB medium at 26°C. The positions of the LamB, OmpC, OmpF, OmpA, and KdgM2 proteins are indicated. MW – molecular weight standards (Thermo Scientific PageRuler Prestained Protein Ladder). The Coomassie blue stained 10% SDS-polyacrylamide gel shown is representative of the results of an experiment performed in triplicate.

and an optimal concentration of phosphorylated OmpR may be required to control the amount of KdgM2 protein. We cannot rule out the possibility that complementation by the *ompR* gene in multicopy leads to an excess of OmpR relative to EnvZ. Thus, the ratio of molecules of the kinase EnvZ to those of the substrate OmpR may be greatly imbalanced, which may influence the expression of *kdgM2*.

Construction and Characterization of *kdgR*-Deficient *Y. enterocolitica* Strains

Data showing upregulation of *kdgM2* caused by the *ompR* mutation and induction of *kdgM2* in the presence of OGAs led us to assume that apart from OmpR, the repressor KdgR inhibits *kdgM2* expression. To dissect these effects and, in addition, to eliminate the problem of the transport of inducing OGAs, we examined OmpR-mediated regulation of *kdgM2* expression in the absence of KdgR. The *kdgR* gene was deleted in both the wild-type strain Ye9N (*kdgR* mutant ES1) and in the *ompR* mutant AR4 (*ompRkdgR* mutant AR11). To study *kdgM2* expression in these strains, the plasmid pBKRFP-Tp carrying the *kdgM2′-′rfp* translational fusion was introduced. The presence of the gentamicin (Gm) resistance cassette in the mutant *kdgR* made it necessary to insert a trimethoprim (Tp) cassette into pBKRFP carrying the *kdgM2′-′rfp* fusion. RFP fluorescence was examined in cultures grown at 26°C in LB (**Figure 3A**) and MMA medium (**Figure 3B**). Data for the strains grown to exponential phase in LB medium (**Figure 3A**) revealed that in strain ES1 the absence of only KdgR in the wild-type background resulted in a 4-fold increase in the expression of *kdgM2* (for comparison a 2.5-fold increase was observed in the wild-type in the presence of OGAs). The absence of only OmpR in strain AR4 resulted in a 1.4-fold increase in *kdgM2* expression. In strain AR11, the lack of KdgR in the *ompR* mutant background resulted in significantly higher *kdgM2* expression than that detected in the strains with single *ompR* (3.9-fold) or *kdgR* (1.2-fold) mutations. The same correlation was obtained with the strains grown in LB to stationary phase (data not shown).

The effect of the *ompR* mutation on *kdgM2* expression was also examined in strains grown in minimal medium to exponential phase. In MMA+glycerol, the upregulation of *kdgM2* in the *ompR*-deficient strain was 4.5-fold, while in the *kdgR*-deficient strain it was 22-fold (**Figure 3B**). In the strain containing both regulatory mutations *ompRkdgR*, the level of *kdgM2* expression was significantly higher (100-fold) than in either single mutant, suggesting a synergistic mode of regulation. These data led us to the hypothesis that this growth condition triggers a cascade of derepression and activation of the *kdgM2* gene, allowing its maximal expression. The addition of OGAs to the minimal medium produced further upregulation of *kdgM2* expression in the *ompR* (30-fold) and *kdgR* (29-fold) strains. The additive effect of *kdgR* and *ompR* mutations was observed in *ompRkdgR* mutant (60-fold) suggested that KdgR and OmpR regulate *kdgM2* expression independently. Complementation of the *kdgR* mutation with the wild-type *kdgR* allele expressed from plasmid pHSG575 caused a very strong reduction in *kdgM2* expression back to the wild-type level. Interestingly, the expression of *kdgR in trans* in the *ompRkdgR* mutant almost completely abolished the positive regulatory effect of the *ompR* mutation.

Taken together, these results confirmed the role of KdgR as a repressor of *kdgM2* expression and showed that OmpR likely contribute to the negative regulation of *kdgM2* expression irrespective of KdgR. The observed induction of *kdgM2* by OGAs in minimal medium in the absence of KdgR suggested that other regulatory factors acting independently of KdgR (also active in the *kdgR* mutant) might be responsible for the induction by intermediates of OGA catabolism. The inducing power of OGAs in the absence of *kdgR* was reported previously in a study analyzing the production of pectate lyase in *kdgR* mutants in the presence of other mutations (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1989). Finally, the medium-dependent expression of *kdgM2* could be the consequence of variations in the respective level/activity of OmpR and KdgR. Previously, differences in *kdgN* porin expression were observed during the growth of *D. dadantii* in rich LB medium and M63 minimal medium, but the underlying regulatory mechanism was not identified (Condemine and Ghazi, 2007).

The Level of KdgM2 Porin in the Outer Membrane of *Y. enterocolitica* Is Influenced by Regulators OmpR and KdgR

We determined the effect of KdgR and OmpR on the KdgM2 protein content in the OM by examining wild-type Ye9 and isogenic strains with *kdgR*, *ompR*, and *ompRkdgR* mutations (**Figure 3C**). The KdgM2 protein band was absent from the gel profile of the wild-type Ye9. strain grown in LB medium and appeared in an OM fraction from cells of *kdgR* mutant ES1 confirmed that KdgR represses expression of this protein. The effect of a lack of OmpR was examined in the presence (*ompR* mutant, strain AR4) and absence (*ompRkdgR* mutant, strain AR11) of a functional *kdgR* gene. The *ompR* mutant AR4 exhibited an increased level of KdgM2 expression compared to the parental wild-type strain Ye9, confirming the observation that initiated our studies and the data from genetic analyses. The level of the KdgM2 in the *ompR* mutant was similar to that observed in the *kdgR* mutant strain. In the double *ompRkdgR* mutant strain AR11, the level of KdgM2 was significantly increased compared to the single *ompR* and *kdgR* mutants (for both ∼1.4-fold). This result suggested that the lack of OmpR increased the biosynthesis of KdgM2 independently of KdgR. When the wild-type allele of *ompR* was introduced into mutant AR4 *in trans* on plasmid pBR3, the production of KdgM2 decreased significantly, although not to the wild-type level. These data demonstrated the negative effect of OmpR in controlling the level of KdgM2 and corroborated the results of the *kdgM2* reporter fusion experiments described above.

Increased Pel Activity in the *ompR* and *kdgR* Mutants

It was previously shown that *Y. enterocolitica* produces a few intracellular pectate lyases, i.e., enzymes involved in OGA degradation, including periplasmic PelP (*Ye*PL2A),

cytoplasmic PelW (*Ye*PL2B) and Ogl (*Ye*OGL) (Abbott and Boraston, 2007, 2008). It has been postulated that *kdgM2, pelP* and *sghX*, encoding porin KdgM2, pectate lyase PelP and putative periplasmic polygalacturonate binding protein SghX, respectively, might be organized in an operon in *Yersiniae* (Rodionov et al., 2004). To confirm this arrangement in *Y. enterocolitica* wild-type strain Ye9, RT-PCR analysis was performed with cDNA using primer pairs specific for the *kdgM2-pelP-sghX* mRNA (**Figure 4A**, upper panel). The results of this analysis showed that these three genes are co-transcribed as a polycistronic mRNA (**Figure 4A**, lower panel). Thus, in addition to porin KdgM2, the production of proteins PelP and SghX might also be regulated by OmpR.

To examine whether the lack of OmpR or KdgR influenced the production of periplasmic pectate lyase PelP by *Y. enterocolitica*, we used the pectate lyase (Pel) plates assay (**Figure 4B**). The presence of PelP lyase in periplasmic fluid (released by osmotic shock) of the wild-type (Ye9), *ompR* mutant (AR4), *kdgR* mutant (ES1) and the *ompRkdgR* mutant (AR11) was tested. In the wild-type Ye9, pectate lyase production was low. In contrast, high pectate lyase activity was observed in the *kdgR* mutant, confirming the repressive activity of KdgR. The *ompR* mutant (AR4) exhibited an increased level of pectate lyase production compared to wild-type cells, which was comparable to that of the *kdgR* mutant strain (ES1). Pectate lyase activity in the double *ompRkdgR* mutant was slightly higher than in the single mutants. Complementation of the *ompR* mutation using pBR3 caused a reduction in PelP activity (**Figure 4B**). The changes in PelP production in the tested *Y. enterocolitica* mutants lacking OmpR, KdgR or both regulatory proteins, reflected the level of porin KdgM2 present in these mutants. These results corroborated our finding that *kdgM2* and *pelP* are organized in an operon whose promoter is negatively regulated by OmpR and KdgR.

OmpR Positively Regulates the Expression of *kdgM1*, a Paralog of *kdgM2*

Bioinformatic analysis revealed the presence of another gene encoding an oligogalacturonide-specific porin of the KdgM family in the genome of *Y. enterocolitica*, i.e., *kdgM1* (**Additional File 1**). To verify whether OmpR or KdgR control *kdgM1* transcription, we examined the expression of a *kdgM1-lacZYA′* chromosomal transcriptional fusion in the wild-type strain (Ye9NK1), *ompR* mutant (AR4K1), *kdgR* mutant (ES1K1) and *ompRkdgR* mutant (AR11K1) (**Figures 5A–C**). First, we measured the β-galactosidase activity in LB medium, in strains differing in their OmpR content, in the absence and presence of OGAs (**Figures 5A,B**). The expression of the transcriptional fusion in cells of the wild-type strain grown in LB medium to stationary phase was very low and it was increased 4.3-fold by the addition of OGAs. In non-inducing conditions, we did not observe any difference between the wild-type strain and the

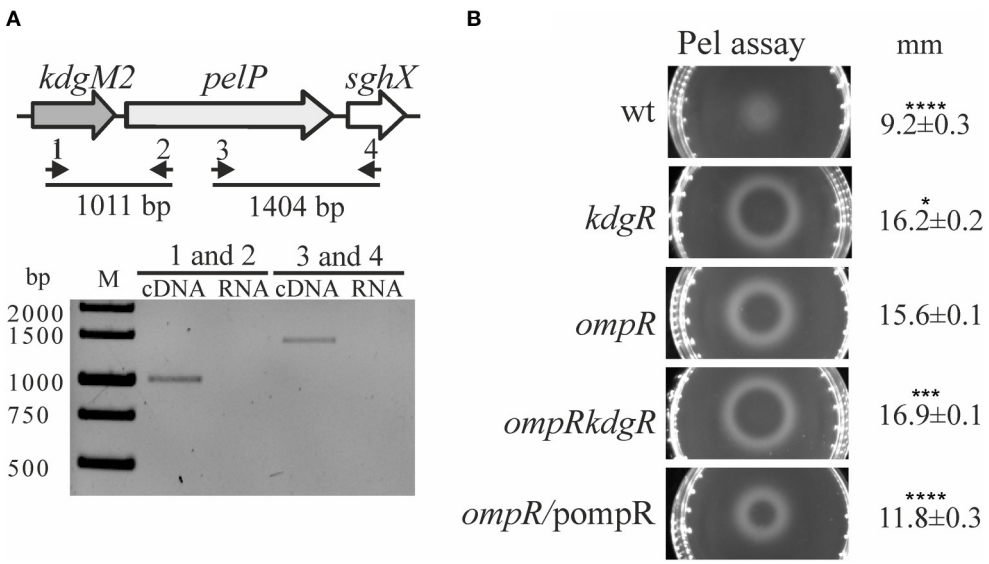

FIGURE 4 | Effect of OmpR and KdgR on pectate lyase (PelP) production. **(A)** To determine whether the *kdgM2, pelP*, and *sghX* genes are organized in an operon, two pairs of primers were used in RT-PCR analysis (1–RTkdgMpelP1/2–RTkdgMpelP2 and 3–RTpelPsghX1/4–RTpelPsghX2) (**A**, upper panel). RT-PCR analysis of the *kdgM2-pelP-sghX* operon (**A**, lower panel). Total RNA isolated from strain Ye9 grown in LB medium at 26°C was DNase treated and then reverse transcribed into cDNA with pairs of primers shown in **(A)** upper panel. M – molecular size marker GeneRuler 1 kb DNA Ladder; bp. RNA was used as the template in negative control reactions. **(B)** Changes in pectate lyase (PelP) production in the tested *Y. enterocolitica* mutants lacking OmpR, KdgR or both regulatory proteins. Periplasmic PelP activity was determined in a semi-quantitative manner by measuring the diameter of haloes on plates containing OGAs and Ca^{2+}, 48 h after inoculation with osmotic shock fluid obtained from exponential phase *Y. enterocolitica* cultures grown in LB medium at 26°C. PelP production was compared in the following strains: Ye9 (wild-type), ES1 (*kdgR* mutant), AR4 (*ompR* mutant), AR11 (*ompRkdgR* mutant), and complemented AR4 (*ompR*/pompR). The data represent mean values with standard deviations, obtained in at least three independent experiments. Significance was calculated using one-way ANOVA (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$). *P*-values were calculated using (assuming unequal variances) comparing test strains to the AR4 strain.

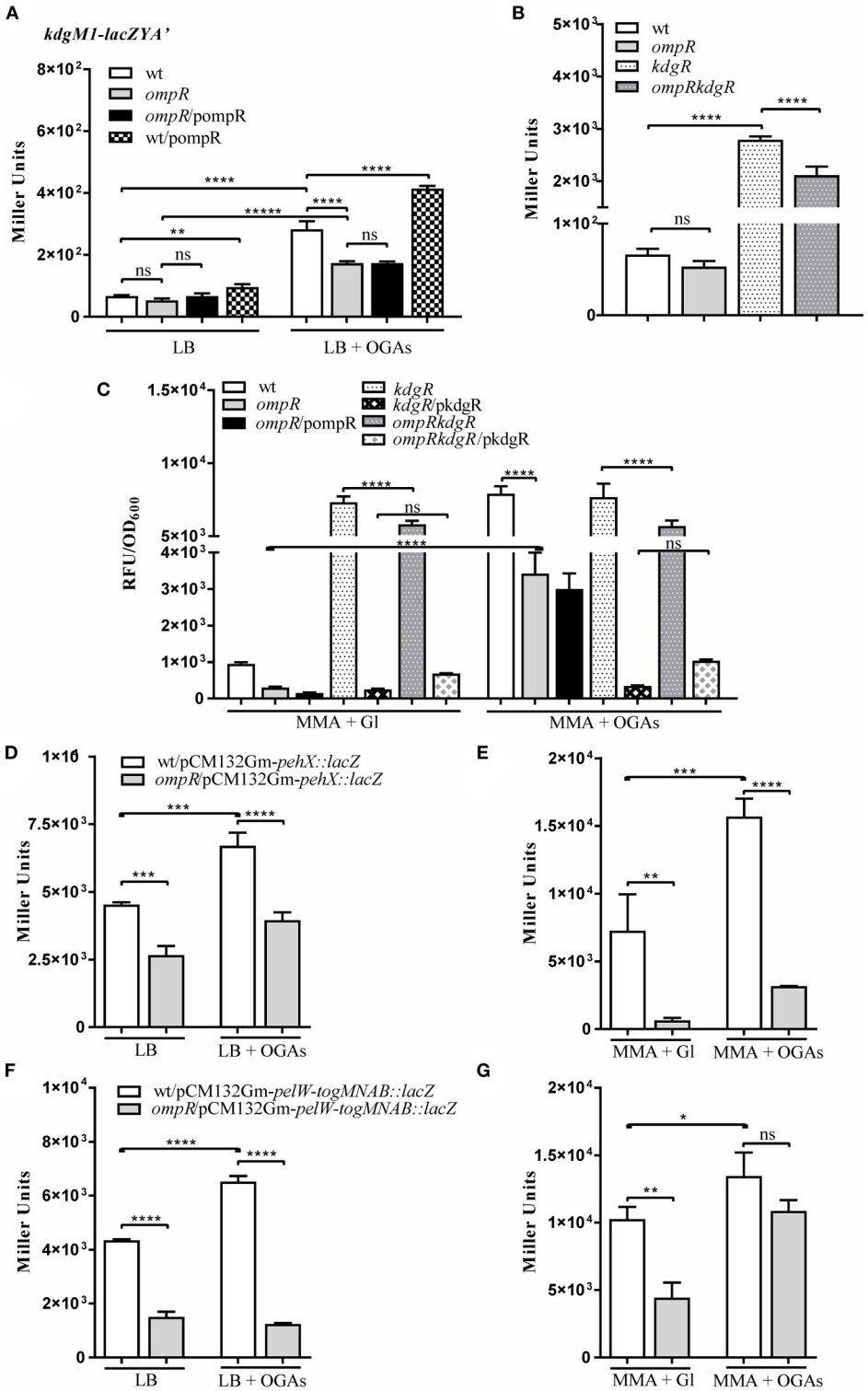

FIGURE 5 | OmpR promotes expression of the genes/operon of the KdgR regulon: *kdgM1* **(A–C)** *pehX* **(D,E)** and *pelW-togMNAB* **(F,G)**. **(A–C)** β-galactosidase activity of strains carrying a chromosomal *kdgM1-lacZYA′* transcriptional fusion, with or without OmpR or/and KdgR: Ye9NK1 (wild-type, wt), AR4K1 (*ompR* mutant), AR4K1/pompR (complemented *ompR* mutant, *ompR*/pompR) and Ye9NK1/pompR (wild-type overproducing OmpR, wt/pompR), ES1K1 (*kdgR* mutant) and AR11K1 (*ompRkdgR* mutant). β-galactosidase activity of Ye9N (wild-type) and AR4 (*ompR* mutant) strains containing *pehX* **(D,E)** and *pelW-togMNAB* **(F,G)** transcriptional fusions with *lacZ* expressed from plasmids pCM132Gm-*pehX::lacZ* and pCM132Gm-*pelW-togMNAB::lacZ*, respectively. Strains were grown at 26°C without or with OGAs, to stationary phase in LB medium **(A,B,D,F)** or to exponential phase in MMA medium **(C,E,G)**. The data represent mean β-galactosidase activity values (Miller units) with the standard deviation from at least two independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using one-way ANOVA (ns [non-significant] $P > 0.05$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

ompR mutant. In LB supplemented with OGAs, the activity of the *kdgM1* promoter in the *ompR* mutant was increased, but to a lesser extent than in the wild-type strain. To confirm the positive OmpR-dependent regulation of *kdgM1,* plasmid pBR3 carrying the wild-type *ompR* allele was used to complement the *ompR* mutation in strain AR4K1. However, complementation did not produce any change in the level of reporter gene expression. We next introduced pBR3 into the wild-type strain (Ye9NK1) and found that overexpression of OmpR caused a 1.5-fold increase in β-galactosidase activity compared to the wild-type strain alone (**Figure 5A**). Notably, the lack of KdgR caused a 42-fold upregulation of the *kdgM1* promoter in strain ES1K1, suggesting that KdgR strongly represses KdgM1 production in LB medium (**Figure 5B**). We could not rule out the possibility that in the absence of the KdgR repressor, putative activators may increase *kdgM1* expression under these growth conditions. In the *ompRkdgR* mutant AR11K1 we observed a significant decrease in reporter gene expression (1.3-fold) compared to the *k*dgR *mutant* (ES1K1). The positive influence of OmpR on *kdgM1* expression was also seen in strains grown to exponential phase in LB medium (data not shown).

To shed light on the OmpR-dependent regulation of *kdgM1* we investigated the expression of the *kdgM1-lacZYA′* fusion in *Y. enterocolitica* strains grown to exponential phase in minimal medium. The 11-fold upregulation of *kdgM1* in the wild-type strain cultured in MMA+OGAs contrasted with the weak expression in MMA+glycerol (**Figure 5C**). Almost the same increase (10-fold) was observed in the *kdgR* mutant independently of the growth conditions (MMA medium supplemented with glycerol or OGAs), confirming the role of KdgR as a repressor of *kdgM1*. In addition, introduction of the wild type *kdgR* allele expressed from plasmid pBBR1MCS-3 reduced *kdgM1* expression in MMA+glycerol. However, the same effect was observed in the presence of the inducer (OGAs). Possibly the intracellular concentration of the direct inducer 2-keto-3-deoxygluconate, a product of OGA degradation, was insufficient to saturate high levels of KdgR expressed from the multicopy plasmid. Inactivation of the *ompR* gene decreased the expression of the *kdgM1* fusion in MMA+glycerol and MMA+OGAs (**Figure 5C**) 2.6-fold and 2.4-fold, respectively, in both the wild-type and the *kdgR* mutant background. These results revealed the role of OmpR in the positive regulation of *kdgM1* expression, independently of the presence of KdgR regulator. Unfortunately, we were unable to detect any complementary effect of the *ompR* mutation. Variations in osmolarity caused no change in the level of *kdgM1* expression examined in exponential phase cultures (data not shown).

OmpR Influences the Expression of Other Members of the KdgR Regulon

To examine whether OmpR might affect the expression of genes of the KdgR regulon in *Y. enterocolitica*, we constructed plasmid-borne *lacZ* transcriptional fusions with the promoter regions of the *pehX* gene encoding the periplasmic polygalacturonase PehX and the *pelW-togMNAB* operon encoding the cytoplasmic exopolygalacturonate lyase PelW and oligogalacturonide transport system TogMNAB (Abbott and Boraston, 2008). The resulting constructs pCM132Gm-*pehX::lacZ* and pCM132Gm-*pelW-togMNAB::lacZ* were introduced into the wild-type strain Ye9N and the *ompR* mutant AR4. Based on β-galactosidase activity measurements, we found that *pehX* and *pelW-togMNAB* expression were both 1.5-fold higher in strain Ye9N grown at 26°C to stationary phase in LB medium supplemented with OGAs, compared to LB alone (**Figures 5D,F**). This confirmed the OGA-inducible nature of both promoters and suggested the participation of KdgR in their repression. The expression of both *pehX* and *pelW-togMNAB* was higher in the wild-type strain compared to the *ompR* mutant AR4 when they were grown in LB medium (1.7-fold and 2.9-fold, respectively) (**Figures 5D,F**), thus revealing the positive impact of OmpR on these genes. In the presence of inducer (OGAs), we still observed reduced *pehX* and *pelW-togMNAB* expression in the *ompR* mutant background. Expression of the *pehX* fusion in strains grown in LB to exponential phase was not significantly modified in the *ompR* mutant, while expression of the *pelW-togMNAB* fusion was equivalent in cells in both the exponential and stationary phases of growth (data not shown). Furthermore, the regulatory role of OGAs and OmpR in the upregulation of the *pehX* (**Figure 5E**) and the *pelW-togMNAB* (**Figure 5G**) was also observed in strains grown to exponential phase in MMA medium without or with OGAs. Interestingly, the OmpR-dependent regulation of the *pelW-togMNAB* expression was not observed in the presence of OGAs. In summary, OmpR seems to be a positive regulator of both studied transcriptional units. However, since no OmpR binding sites have been detected in either the *pehX* or the *pelW-togMNAB* regulatory regions, we presume that the positive influence of OmpR might result from some indirect effect that it exerts on their transcription.

OmpR Negatively Regulates *kdgR* Transcription

To investigate the possible link between the OmpR and KdgR regulatory proteins, OmpR-dependent activity of the *kdgR* promoter was studied using a transcriptional fusion with a promoterless *lacZ* gene, constructed in plasmid pCM132Gm. The resulting construct pCM132Gm-*kdgR::lacZ* was introduced into the wild-type strain Ye9N and the *ompR* mutant AR4. The levels of expression of *kdgR*::*lacZ*, based on measurements of β-galactosidase activity, were determined for both strains grown to exponential and stationary phase in LB medium at 26°C (**Figure 6A**). The activity of the *kdgR* promoter in the *ompR* mutant was 1.9-fold (in exponential phase) and 1.5-fold (in stationary phase) higher than in the Ye9N strain. For complementation analysis, vector pBR3 carrying the entire *ompR* coding sequence was introduced into strain AR4/pCM132Gm-*kdgR::lacZ*. The introduction of *ompR in trans* restored *kdgR* expression to the wild-type level. These results suggested that OmpR negatively regulates *kdgR* expression. Data from the *kdgR::lacZ* reporter fusion were validated by evaluating the expression of *kdgR* in the wild-type Ye9 and the *ompR* mutant AR4 grown to stationary phase in LB+OGAs at 26°C, using quantitative real-time PCR (**Figure 6B**). In addition,

as a control *kdgM2* and *pelP* transcript abundance was also assessed. Transcription of *kdgR* was up-regulated in the *ompR* mutant (3-fold), confirming that OmpR functions as a negative regulator of *kdgR*. In contrast, *kdgM2* and *pelP* were highly up-regulated in the *ompR* mutant, 39-fold and 27-fold, respectively. These results are consistent with those obtained for the *ompR* mutant with the *kdgM2::lacZ* reporter fusion and PelP activity test.

Taken together, these results demonstrated that besides its role in regulating *kdgM2*, *kdgM1, pehX* and *pelW-togMNAB* (shown above), OmpR modulates the expression of their regulator KdgR. Bearing in the mind that OmpR negatively regulates *kdgR* expression, the KdgM2 level may reflect the dual function of OmpR: having both a direct negative effect on *kdgM2* transcription and an indirect effect by repressing *kdgR*. The positive regulatory effect of OmpR on *kdgM1* may result from both its direct positive effect on *kdgM1* expression and the indirect effect of its inhibition of *kdgR* expression.

Putative KdgR and OmpR Binding Sites in the Promoter Regions of *kdgM1*, *kdgM2-pelP-sghX* and *kdgR* of *Y. enterocolitica*

Putative KdgR and OmpR binding sites in the regulatory regions of selected KdgR regulon members were identified by *in silico* analysis (**Figure 7**). The promoters of the *kdgM1*, *kdgM2-pelP-sghX* and *kdgR* genes/operon were initially characterized using BPROM software. Then a search conducted using the *D. dadantii* consensus KdgR binding site sequence (AAATGAAACAnTGTTTCATTT, Rodionov et al., 2000, 2004) led to the identification of potential KdgR-binding site K1 close to the *kdgM1* promoter (90% identity to consensus) and K2 overlapping the *kdgM2-pelP-sghX* promoter (65% identity to consensus). No putative KdgR-binding sites were identified in the *kdgR* regulatory region. The consensus OmpR-binding sequence of *E. coli* (TTTTACTTTTTG(A/T)AACATAT, Maeda et al., 1991) was used to search for putative OmpR binding sites. Two predicted OmpR-binding sites were found in the *kdgM1* promoter region: O1 and O2, both with 55% identity to the consensus (**Figures 7A,B**). The putative O1 and O2 sites are respectively located between nucleotides −277 and −297, and −101 and −121 bp, upstream of the *kdgM1* ATG, with the second site overlapping the proposed KdgR-binding site. The location of this OmpR-binding site might be related to the role of OmpR as an antirepressor in the positive regulation of *kdgM1* transcription. One putative OmpR-binding site was detected in the *kdgM2-pelP-sghX* regulatory sequence: O3 with 60% identity to the consensus (**Figures 7A,B**), located between nucleotides −141 and −161 bp upstream of the *kdgM2* ATG. The results of this analysis indicated that *kdgM1* and *kdgM2-pelP-sghX* might be subject to dual regulation by KdgR and OmpR in *Y. enterocolitica*. Two potential OmpR-binding sites were also recognized in the *kdgR* regulatory region: sites O4 and O5 located between nucleotides −207 and −227 bp and −24 and −44 bp upstream of the *kdgR* ATG, with 50% and 65% identity to the consensus, respectively (**Figures 7A,B**). The results of Logo motif analysis of these putative *Y. enterocolitica* OmpR-binding sites are shown in **Figure 7C**. The presence of OmpR-binding

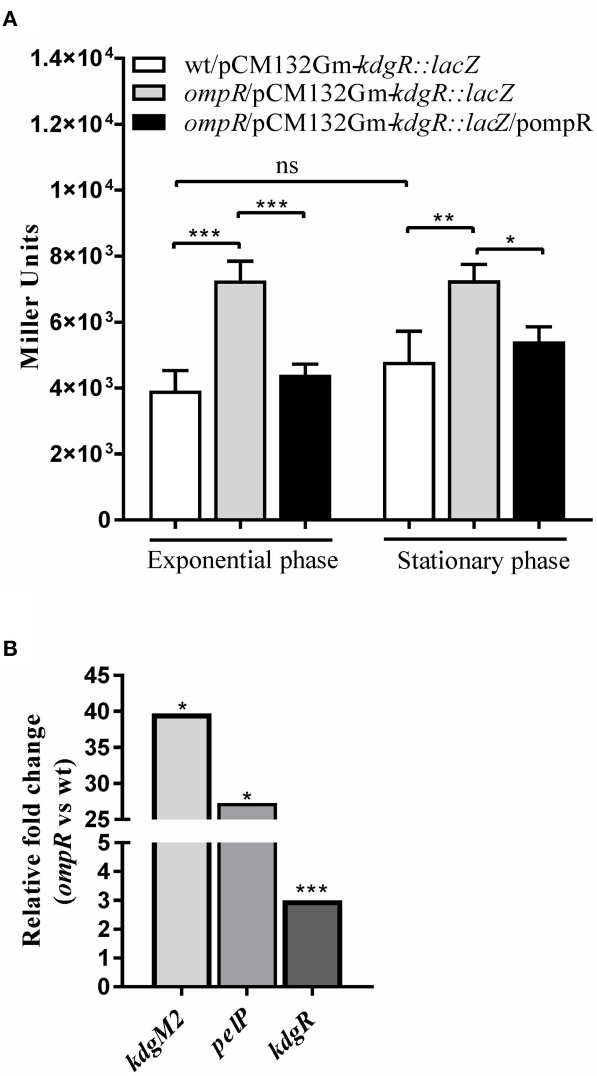

FIGURE 6 | Effect of OmpR on *kdgR* expression using *lacZ* reporter fusions **(A)** and RT-qPCR analysis **(B)**. **(A)** β-galactosidase activity of strains Ye9N (wild-type), AR4 (*ompR* mutant) and complemented AR4 (*ompR*/pompR) containing a *kdgR::lacZ* transcriptional fusion expressed from plasmid pCM132Gm-*kdgR::lacZ*. Strains were grown to exponential and stationary phase in LB at 26°C and β-galactosidase activity was assayed. The data represent mean activity values (Miller units) with the standard deviation from two independent experiments, each performed using at least triplicate cultures of each strain. Significance was calculated using one-way ANOVA (ns [non-significant] $P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). **(B)** mRNA levels of *kdgR*, *kdgM2* and *pelP* were determined by quantitative real-time polymerase chain reaction (RT-qPCR analysis) from wild-type and *ompR* mutant strains grown in LB+OGAs to stationary phase at 26°C as described in Materials and Methods. Representative results from two independent experiments, performed in quadruplicate, are shown. The statistical significance of differences in transcripts of tested genes were analyzed using Student's *t*-test. Stars indicate statistically significant differences ($^{*}P < 0.05$, $^{***}P < 0.001$).

A

kdgM1 promoter region

```
                          O1
GTTAATAAATTAATTTAACTTAAGTATCTATTAAACCCTGCCATTAATTTGGCGGGGTTCTTTTGTTTTATAGCCATAA

AATAATAACCAACCAGACAGACATCACAATTTAATATCTGTTTAAAAAAAGAATCATTAAGACAGATCTGGATCACATA

                                    O2        K1                          -35
AATATTTAATAATATAAATAGAATATGTATTCACAATAAATGAAACATTGTTTCTTTGTTGTCGGAATTGTTTTTCACT

                -10                                                  SD          >kdgM1
CGTGTGTTGATTTTTTATAGATTTGTTAATGACTTTTGATTTAAATAACCATAACCCTGGGATTATATAAAAATG
```

kdgM2-pelP-sphX promoter region

```
                    O3
ACTATTTACTTTGAACCTTGTCACATAAATGGATATTGGCTCTATTAATTTCTAACTATTAGTCATGGATCTCGGAACT

                        K2      -35                  -10
TTATATCAAAATAAATAGAATGGCGTTTCATAAGTGAGATCTATATTTATCATAATAATTAATGTTATTTATTAAAATA

 SD          >kdgM2
AGGTAACTGTAATG
```

kdgR promoter region

```
                          O4                                    >ogl
CACGGTAAACCAGCACTATATTTAGCCACATTGCCTGAATCAGTCTGGAAATAGTTGAATTTTGCACTAGCCTCTTTCA

                                                          -35
TGGAAAGAGGCTAATTTGCTGTCTGCAAGGCTTTTATCCGATTATTCACATTGGTTTATTGCACAGTCTCGCGACTTCC

    -10                                   O5                          SD
CTTTATAATAAAACCACTGTTCTATTTTTTTTAAAACAAATTGTTTTATGAAACACAGAAAACTGAGTAGGGTAACCAA

    >kdgR
AAAATG
```

B

Putative OmpR-binding sites

Gene		Binding Motif	% identity to consensus *E.coli*
O1	*kdgM1*	ATTTAACTTAAGTATCTATT	55
O2	*kdgM1*	TCACAATAAATGAAACATTG	55
O3	*kdgM2*	CTTTGAACCTTGTCACATAA	60
O4	*kdgR*	CACTATATTTAGCCACATTG	50
O5	*kdgR*	AATTGTTTTATGAAACACAG	65
E. coli consensus		TTTTACTTTTTG(A/T)AACATAT	

C

FIGURE 7 | Putative OmpR-binding sites in the promoter regions of the *kdgM1*, *kdgM2-pelP-sghX* and *kdgR* genes of *Y. enterocolitica*. **(A)** The boxes indicate putative −35 and −10 promoter elements. The SD sequence is underlined. The start codon (ATG) of each gene is shown in bold. Putative OmpR binding sites identified by *in silico* analysis (O1-O5) are boxed. Potential binding sites for KdgR (K1, K2) are shaded gray. **(B)** Sequences of putative OmpR-binding sites in the promoters of the indicated *Y. enterocolitica* genes determined based on similarity to the *E. coli* consensus sequence (% identity values are shown). The central motif GXXAC or GXXXC and the AC or C nucleotides usually located about 10 nt away from the AC elements of the central motif are marked. **(C)** Logo motif (alignment) of putative OmpR-binding sites identified in the *Y. enterocolitica kdgM1*, *kdgM2-pelP-sghX* and *kdgR* regulatory regions. WebLogo (http://weblogo.berkeley.edu/logo.cgi) was used to obtain consensus sequence logos in which the height of individual letters represents the relative frequency of that particular nucleotide at a given position, and the number of letters in each stack indicates the degree of conservation at that position.

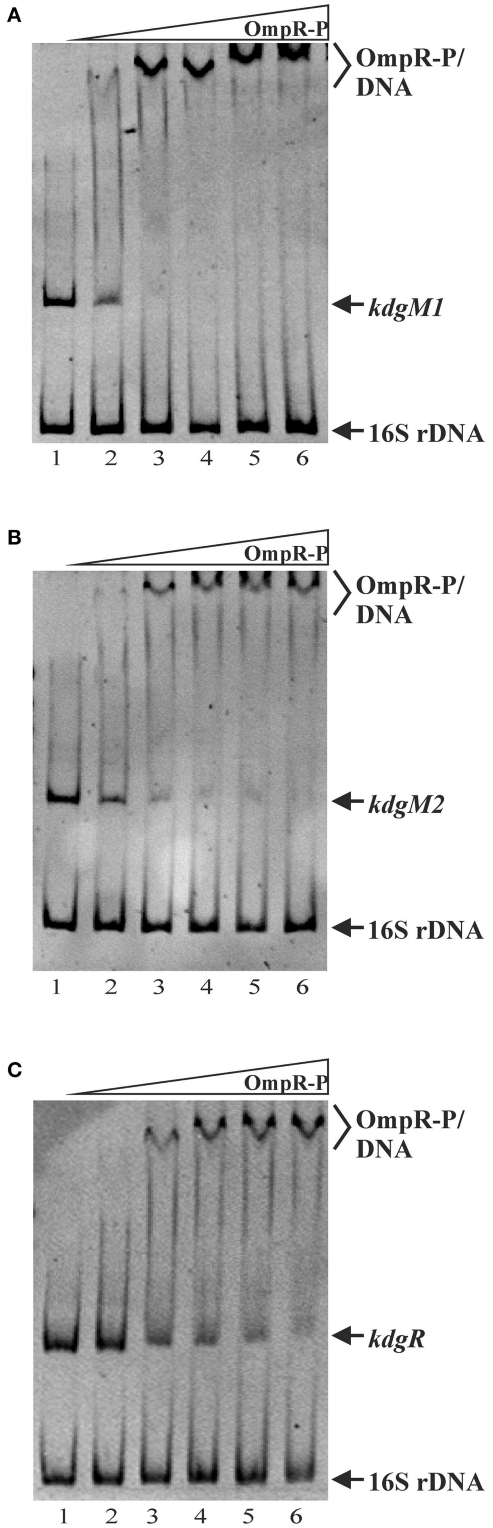

FIGURE 8 | Binding of phosphorylated OmpR to the *kdgM1*, *kdgM2-pelP-sghX*, and *kdgR* regulatory regions examined using EMSAs. A range of concentrations of OmpR-P were incubated with DNA fragments representing the *kdgM1* (**A**, 544 bp), *kdgM2-pelP-sghX* (**B**, 500 bp) and *kdgR* (**C**, 441 bp) promoters which contain putative OmpR-binding sites.

(Continued)

FIGURE 8 | Continued

A fragment of 16S rDNA (304 bp) was included in each reaction mixture as a non-specific binding control. The DNA fragments were mixed with increasing concentrations of OmpR-P in lanes 1–6: 0, 0.167, 0.333, 0.500, 0.583, 0.667 μM. The identities of the bands resolved by electrophoresis on native 5% polyacrylamide gels are indicated.

sites in the *kdgR* regulatory region suggests a direct role for OmpR in regulating KdgR expression.

OmpR Directly Regulates *kdgM1, kdgM2*, and *kdgR* Expression in *Y. enterocolitica*

As shown above, *in silico* analysis led to the identification of putative OmpR-binding sites in the promoter regions of the *Y. enterocolitica kdgM1* gene (2 sites), *kdgM2-pelP-sghX* operon (1 site) and *kdgR* gene (2 sites) (**Figure 8**). To verify whether OmpR directly binds to these sequence elements, electrophoretic mobility shift assays (EMSAs) were performed. A recombinant OmpR-His_6 protein was expressed in *E. coli*, purified to homogeneity and phosporylated *in vitro* using acetyl-phospate. It was shown previously that OmpR can be phosphorylated by acetyl phosphate *in vivo* and *in vitro* (Shin and Park, 1995). DNA fragments representing the promoter region of the analyzed genes/operon, containing the putative OmpR-binding sites, and a fragment of 16S rDNA, as a non-specific binding control, were incubated with different amounts of the OmpR-P and these binding reactions were analyzed by electrophoresis in non-denaturing 5% polyacrylamide gels. As shown in **Figure 8**, OmpR-P was able to bind the upstream regions of *kdgM1*, *kdgM2-pelP-sghX*, and *kdgR*, but not the 16S rDNA fragment. Shifted complexes were clearly produced by interaction between the *kdgM1* and *kdgM2-pelP-sghX* fragments and phosphorylated OmpR present at a concentration of 0.167 μM (**Figures 8A,B**). Interestingly, the OmpR-P protein interacted with the *kdgR* promoter fragment with slightly lower affinity. A slower migrating nucleoprotein band appeared when a higher concentration of OmpR-P (0.333 μM) was incubated with the *kdgR* fragment (**Figure 8C**). Since our *in silico* analysis identified two putative OmpR-binding sites in the regulatory regions of *kdgM1* and *kdgR*, a stepwise shift in the nucleoprotein complexes might be expected in EMSAs. Indeed, we observed a slight stepwise shift when both promoter region fragments were used in an EMSA. To reveal the exact number of OmpR binding sites and the specific nucleotide sequence to which OmpR-P binds, a DNase I footprinting experiment would be necessary. Taken together, these results demonstrated that OmpR can specifically bind to the *kdgM1*, *kdgM2-pelP-sghX*, and *kdgR* promoter regions.

Intracellular ROS Production in Response to Short OGAs

Short oligogalacturonides (sOGAs) penetrating the cells of phytopathogens are able to induce the generation and accumulation of reactive oxygen species (ROS) (Côté and Hahn, 1994; Ridley et al., 2001). sOGAs have been shown to evoke a transient accumulation of ROS in *Rhizobium leguminosarum* bv. *viciae* 3841 (Moscatiello et al., 2012). To investigate the effect of

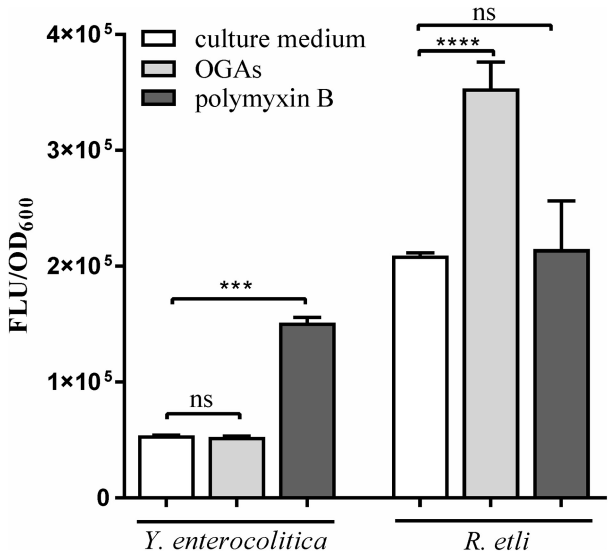

FIGURE 9 | Effect of short oligogalacturonides (sOGAs) and polymyxin B on the production of ROS in *Y. enterocolitica* Ye9 and *R. etli* CE3. ROS formation was detected using the fluorescent dye H2DCF-DA. Strains were cultivated in LB medium (*Y. enterocolitica*) or in TY medium (*R. etli*) at 26°C to exponential phase and treated with sOGAs (50 μg/ml) or polymyxin B (25 μg/ml). Data represent mean fluorescence activity values normalized to the OD_{600} of the culture (± standard deviation) from experiments performed in triplicate. Significance was calculated using one-way ANOVA [ns (non-significant) $P > 0.05$, ***$P < 0.001$, ****$P < 0.0001$].

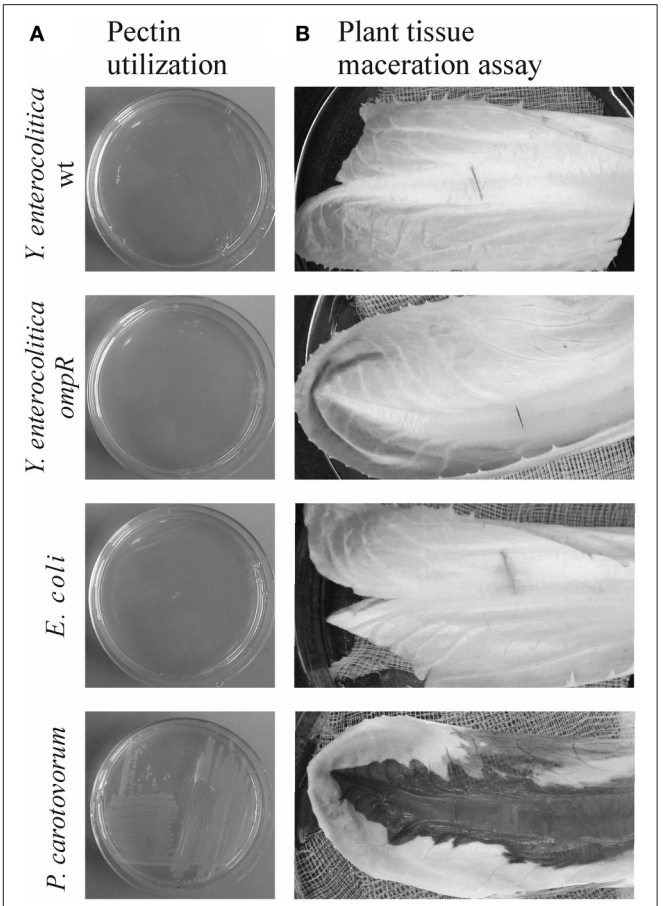

FIGURE 10 | Pectin utilization **(A)** and plant tissue maceration assay **(B)**. **(A)** The ability of *Y. enterocolitica* strains to utilize pectin was assessed visually by analyzing the growth of strains on MMA plates supplemented with 2% (w/v) pectin after incubation at room temperature for 48 h. **(B)** Aliquots of 10 μl of each bacterial suspension (~10^8 cells) were injected into chicory leaves. The inoculated leaves were incubated in a moist chamber at room temperature for 24 h and then examined for evidence of tissue maceration. The following bacterial strains were tested in this assay: *Y. enterocolitica* wild-type Ye9 and *ompR* mutant AR4, *P. carotovorum* PCM 2056 (positive control) and *E. coli* W (negative control).

sOGAs on cells of *Y. enterocolitica* Ye9 and *Rhizobium etli* CE3 we analyzed intracellular ROS production following treatment with sOGAs by measurement of 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescence (**Figure 9**). Short linear α-1,4-linked oligogalacturonide molecules were obtained by polygalacturonase digestion of PGA. It was recently shown that polymyxin B, an antimicrobial peptide that attacks the cell envelope, induces oxidative stress in *E. coli* (Dong et al., 2015). Therefore as a control, we measured ROS production by *Y. enterocolitica* Ye9 and *R. etli* CE3 caused by polymyxin B.

In comparison with the untreated control, sOGAs induced an almost 2-fold increase in ROS level in *R. etli* CE3 cells, but this effect was not observed in the case of *Y. enterocolitica* Ye9 (**Figure 9**). Conversely, we found no polymyxin B-induced effect on the production of ROS in *R. etli* CE3, but treatment with this antimicrobial agent caused an accumulation of ROS in *Y. enterocolitica* Ye9 (**Figure 9**). In contrast to polymyxin B, sOGAs appeared to have no effect on the production of ROS in *Y. enterocolitica*.

Pectin Utilization and Plant Tissue Maceration by *Y. enterocolitica* Strains

Strains of *Y. enterocolitica* differing in their OmpR content were examined for their ability to grow on plates supplemented with 2% pectin (**Figure 10A**) and macerate chicory leaves (**Figure 10B**). While *P. carotovorum* PCM 2056 was able to grow on the pectin plates, no visible growth of *Y. enterocolitica* strain Ye9 or the *ompR* mutant AR4 was observed. When *P. carotovorum* PCM 2056 was injected into chicory leaves, a black, macerated lesion developed within 12 h. Neither wild-type Ye9 nor the *ompR* mutant AR4 was capable of leaf tissue maceration in comparison with *P. carotovorum* (**Figure 10B**). This lack of maceration ability was also observed in *kdgR* and *ompRkdgR* mutants of *Y. enterocolitica* (data not shown). *E. coli,* which lacks OGA transport proteins and pectinases was applied as a negative control and was also incapable of tissue maceration.

Antimicrobial Susceptibility Testing

We measured the antibiotic susceptibility of wild-type *Y. enterocolitica* Ye9 and different mutant strains using the broth micro-dilution test. The *ompR* mutant strain AR4 (lacking OmpC and OmpF porins, with strong upregulation of KdgM2)

TABLE 1 | Antibiotic susceptibility of *Y. enterocolitica* strains.

Antibiotic	MIC (μg/ml)*			
	wild-type (OmpC/F$^+$, KdgM1$^-$/2^{-**})	*kdgR* (OmpC/F$^+$, KdgM1$^+$/M2$^+$)	*ompR* (OmpC/F$^-$, KdgM1$^-$/2$^+$)	*ompRkdgM2* (OmpC/F$^-$, KdgM1$^-$/2$^-$)
Ampicillin	100	100	400	400
Cefotaxime	0.063	0.063	0.125	0.125
Ceftazidime	0.031	0.031	0.25	0.25
Cephaloridine	1.953	1.953	500	500
Cephalothin	15.625	15.625	500	500
Chloramphenicol	3.125	3.125	3.125	3.125
Tetracycline	0.313	0.313	0.625	0.625

**Three independent replicates gave an identical MIC value. **The production of porins KdgM1 and KdgM2 is inhibited in LB medium.*

showed reduced sensitivity to the tested β-lactam antibiotics and tetracycline (**Table 1**). However, the antibiotic susceptibility of the *ompRkdgM2* double mutant strain AR10 was not markedly different from that of the *ompR* mutant. These data implied that the resistant phenotype correlated with the loss of the general porins OmpC and OmpF (confirming our previous observation, Brzostek and Raczkowska, 2007) and that KdgM2 is not a significant entry route for antibiotics. The finding that the null *kdgR* mutation (upregulation of both KdgM1 and KdgM2 porins) did not render *Y. enterocolitica* cells more sensitive to any of the tested antimicrobial compounds supports this conclusion.

Porin KdgM2 Enhances the Outer Membrane Permeability in *Y. enterocolitica*

To address the possibility that the porin KdgM2 can influence outer membrane permeability of *Y. enterocolitica*, an NPN accumulation assay was applied. The use of NPN to study the structure and function of biological membranes is well documented (Hancock, 1984; Loh et al., 1984). NPN, a neutral hydrophobic fluorescent probe, is normally excluded by the outer membrane but exhibits increased fluorescence intensity when it partitions into this membrane. This assay was performed for wild-type strain Ye9, the *kdgR* mutant ES1, the *ompR* mutant AR4 and the *ompRkdgM2* double mutant AR10. Given the influence of OmpR on the outer membrane protein composition, particularly affecting levels of porin KdgM2 (**Figure 2A**), we also used this assay to examine a strain overexpressing KdgM2. The *kdgM2* coding sequence was cloned into the high-copy-number vector pBAD18Km to generate pBAD-KdgM2 and this was introduced into the wild-type strain Ye9. Induction with L-arabinose led to overexpression of KdgM2 that was detected by SDS-PAGE analysis (**Figure 11A**). Preliminary experiments were carried out using a growth temperature of 26°C (optimal for *Y. enterocolitica*), but we saw no change in NPN fluorescence in any of the studied strains (data not shown). However, when the assay was performed at 37°C, clearly visible changes were apparent (**Figure 11B**). The greatest increase in NPN fluorescence was observed in the Ye9 strain overexpressing KdgM2 ($^{**}P < 0.01$). In contrast, we observed no meaningful difference in fluorescence between the wild-type strain, *ompR*, *kdgR* and double *ompRkdgM2* mutants. These data suggested that overexpression of KdgM2 increases the outer membrane permeability in *Y. enterocolitica*.

Susceptibility of *Y. enterocolitica* Strains to Hydrophobic Antimicrobial Compounds and Detergents

The enhanced outer membrane permeability caused by raised levels of KdgM2 (**Figure 11**), prompted us to perform antimicrobial susceptibility assays using the hydrophobic antibiotic gentamicin and trimethoprim (Delcour, 2009). We examined the level of gentamicin and trimethoprim resistance of the wild-type strain Ye9 and Ye9 overexpressing KdgM2 grown at 26°C and 37°C. L-arabinose induction of pBAD-KdgM2 in the wild-type strain Ye9 grown at 37°C significantly enhanced sensitivity to trimethoprim (MIC 3.125 μg/ml) while this did not occur in the absence of the inducer or in the wild-type strain control, at both temperature (MIC 12.5 μg/ml). The susceptibility to gentamicin was not changed in all studied variants.

The role of porin KdgM2 was further explored using a detergent sensitivity assay. The growth of strains differing in their OmpR, KdgR, and KdgM2 contents was assessed in the presence of two detergents: cationic CTAB and anionic SDS (Zou et al., 2011). The wild-type Ye9 was able to grow in up to 200 SDS μg/ml and 1.56 μg/ml CTAB. In comparison, the *ompR* mutant AR4 displayed only a slight increase in sensitivity to SDS (100 μg/ml), while the overexpression of KdgM2 in the wild-type background did not render the cells more sensitive to either of the tested detergents. These findings indicated that the anionic nature of SDS may be a key determinant of its antimicrobial activity against the *ompR* mutant strain.

DISCUSSION

Comparative proteomic analysis suggested that the production of *Y. enterocolitica* outer membrane protein KdgM2, related to oligogalacturonide (OGA)-specific porins of *D. dadantii,* might be negatively regulated by OmpR (Nieckarz et al., 2016). The use of SDS-PAGE analysis followed by mass spectrometry in the

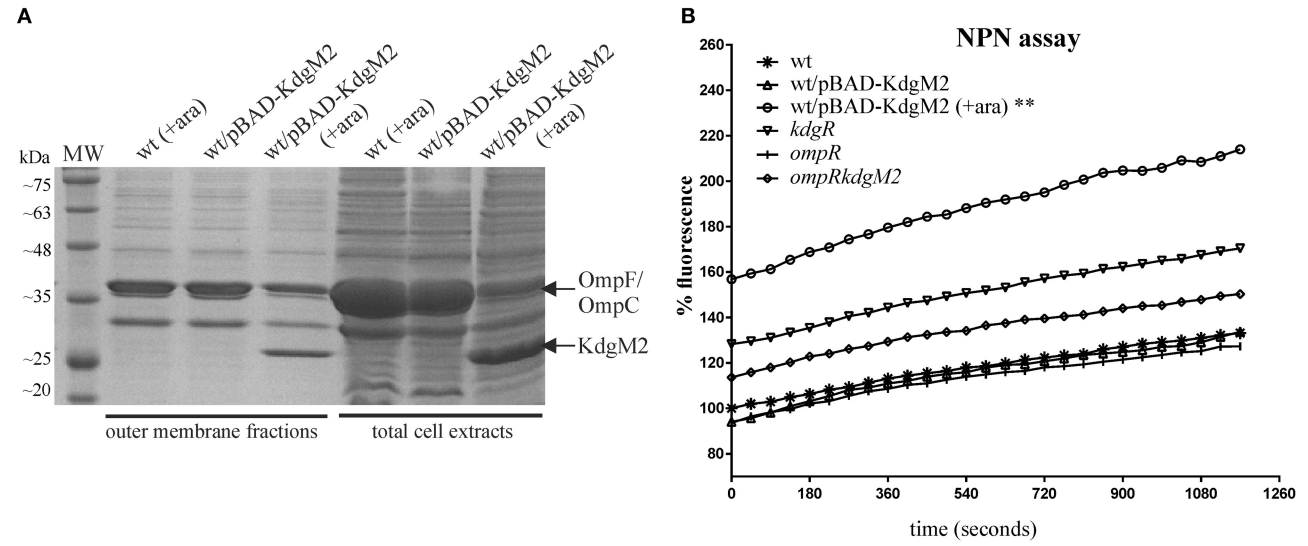

FIGURE 11 | Overexpression of *kdgM2* in *Y. enterocolitica* leads to increased production of KdgM2 in the OM **(A)** and membrane permeability to NPN **(B)**. **(A)** Coomassie blue-stained SDS-PAGE gel shows overproduction of KdgM2 analyzed in OM fractions prepared from bacterial cells grown in LB to exponential phase (OD_{600} ~ 0.5) and in total cell extracts (overnight cultures). Cultures of the wild-type strain Ye9 and Ye9 carrying pBAD-KdgM2 were induced with 0.2% L-arabinose for 1 h (OM fractions) or overnight (total extracts) at 26°C. A non-induced control culture of Ye9 carrying pBAD-KdgM2 was processed in parallel. The KdgM2 protein band is marked. MW – molecular weight standards (3-Color Prestained Protein Marker, DNA-Gdańsk). **(B)** Fluorescent dye NPN uptake by wild-type strain Ye9, strain Ye9/pBAD-KdgM2 induced with L-arabinose to overexpress *kdgM2,* non-induced Ye9/pBAD-KdgM2, *kdgR* mutant ES1 (*kdgR*), *ompR* mutant AR4 (*ompR*) and the *ompRkdgM2* double mutant AR10 (*ompRkdgM2*). Values are averages of three biological replicates. *P*-values were calculated using a one-way ANOVA comparing test strains to the wild-type strain at 19.5 min (**$P < 0.01$).

present study confirmed the strong negative effect exerted by OmpR on the level of KdgM2.

The *kdgM* and *kdgN* genes of *D. dadantii* were shown to be regulated by KdgR, a local repressor of operons/genes involved in pectin catabolism (Nasser et al., 1992; Rodionov et al., 2004). Since the KdgR protein of *Y. enterocolitica* exhibits high sequence similarity (88%) to the corresponding regulatory protein of *D. dadantii*, we reasoned that it should play a similar role in the regulation of *kdgM1* and *kdgM2* expression in *Y. enterocolitica*. Our data showed an increase in the expression of both genes in the *Y. enterocolitica kdgR* deletion mutant, confirming the repression normally exerted by KdgR. In addition, the role of OGAs as an inducer of *kdgM1* and *kdgM2* expression was noted. Induction of OGA-specific porins in the presence of pectin derivatives in *D. dadantii* mainly results from the interaction of the KdgR repressor with an intracellular OGA catabolite such as 2-keto-3-deoxygluconate (KDG) (Hugouvieux-Cotte-Pattat et al., 1996). Since *Y. enterocolitica* can grow on OGAs as the sole carbon source, we speculate that OGA catabolism involves a stage in which KDG is formed. An *in silico* search of the regulatory regions of the *Y. enterocolitica kdgM1* and *kdgM2* genes using the consensus KdgR binding site sequence of *D. dadantii* identified highly similar sequence elements.

The results of reporter gene fusion assays demonstrated that the expression of *kdgM2* is inhibited by OmpR. The additive effect of *kdgR* and *ompR* mutations revealed that KdgR and OmpR regulate *kdgM2* expression independently. Analysis of *kdgM1* expression suggested the positive role of OmpR irrespective of the presence of KdgR. Thus, the expression of *kdgM1* and *kdgM2* appear to be regulated in an inverse manner by OmpR: the former is activated while the latter is repressed. It was previously shown that the production of KdgM and KdgN in *D. dadantii* is also subject to inverse regulation by OmpR, but direct involvement of this regulator in this process has not been reported (Condemine and Ghazi, 2007). In addition, OmpR of *D. dadantii* was shown to repress the expression of *kdgM* located downstream of the *pelW-togMNAB* operon, but we found that OmpR of *Y. enterocolitica* produces the opposite regulatory effect (upregulation) on the homolog *kdgM1*, located in the same genetic context. OmpR has the reciprocal effect on the expression of *kdgN* of *D. dadantii*, i.e., activation, whereas its *Y. enterocolitica* homolog *kdgM2* is repressed by this regulator.

We found that the expression of both *kdgM* genes are inhibited by high osmolarity, although in an OmpR-independent manner. It may be speculated that other regulatory factors or mechanisms might contribute to the modulation of *kdgM1* and *kdgM2* transcription in response to varying osmolarity, as has been reported for some enterobacterial genes (Higgins et al., 1988). Interestingly, some genes regulated by EnvZ/OmpR are not sensitive to osmotic change. The *tpp* genes from *Salmonella enterica* sv Typhimurium and *E. coli* are notable examples (Gibson et al., 1987; Goh et al., 2004). Osmoregulation of *kdgN* expression in *D. dadantii* has been reported previously, and a marginal role for OmpR in this phenomenon was proposed (Condemine and Ghazi, 2007).

In search of other OmpR-regulated genes of the pectinolytic pathway, we performed a bioinformatic analysis to identify putative OmpR-binding sites within regulatory regions.

Unexpectedly, the *in silico* analysis of the 5′ untranslated region of *kdgR* revealed the presence of two putative OmpR-binding sequences. Reporter gene fusion assays and RT-qPCR demonstrated that the expression of KdgR is negatively regulated by OmpR.

Besides the *Y. enterocolitica kdgR* gene, putative OmpR-binding sites were detected in the promoter regions of the *kdgM2-pelP-sghX* operon (1 site) and *kdgM1* gene (2 sites). In addition, single putative KdgR-binding sites were identified in the promoter regions of the *kdgM2-pelP-sghX* operon and *kdgM1* gene. The OmpR- and KdgR-binding sites in the *kdgM2* promoter region are separated by 63 bp. In the *kdgM1* regulatory region, one of the two putative OmpR-binding sites overlaps the KdgR-binding site, suggesting that OmpR bound at this site could act as an antirepressor by preventing KdgR binding. However, the results of reporter gene fusion assays demonstrated a positive role for OmpR in *kdgM1* expression and indicated that OmpR and KdgR act independently to regulate this gene. EMSAs confirmed the binding of *Y. enterocolitica* OmpR to regulatory region fragments of the *kdgM2-pelP-sghX* operon, and the *kdgM1* and *kdgR* genes. This is strong evidence of a direct role for OmpR in the regulation of KdgM2, KdgM1, and KdgR biosynthesis.

The production of porins KdgM1 and KdgM2 is likely to be influenced directly and indirectly by OmpR. The positive regulation of *kdgM1* by OmpR may result from both its direct positive effect on *kdgM1* expression and the indirect effect of its inhibition of *kdgR* expression. The observed strong negative regulation of *kdgM2* by OmpR suggests that the direct inhibitory effect it exerts on this gene is more powerful than any upregulation caused by OmpR-dependent inhibition of *kdgR* expression. However, we cannot rule out the possibility that additional, as yet uncharacterized regulators might exist that are involved in the regulation of *kdgM1* and *kdgM2* in *Y. enterocolitica*. The transcriptional regulatory network controlling the *kdgM* and *kdgN* genes of *D. dadantii* is highly complex and composed of several regulatory proteins, i.e., KdgR, PecS, HNS, OmpR and CRP as well as their cross-regulatory interactions (Blot et al., 2002; Condemine and Ghazi, 2007; Sepulchre et al.,

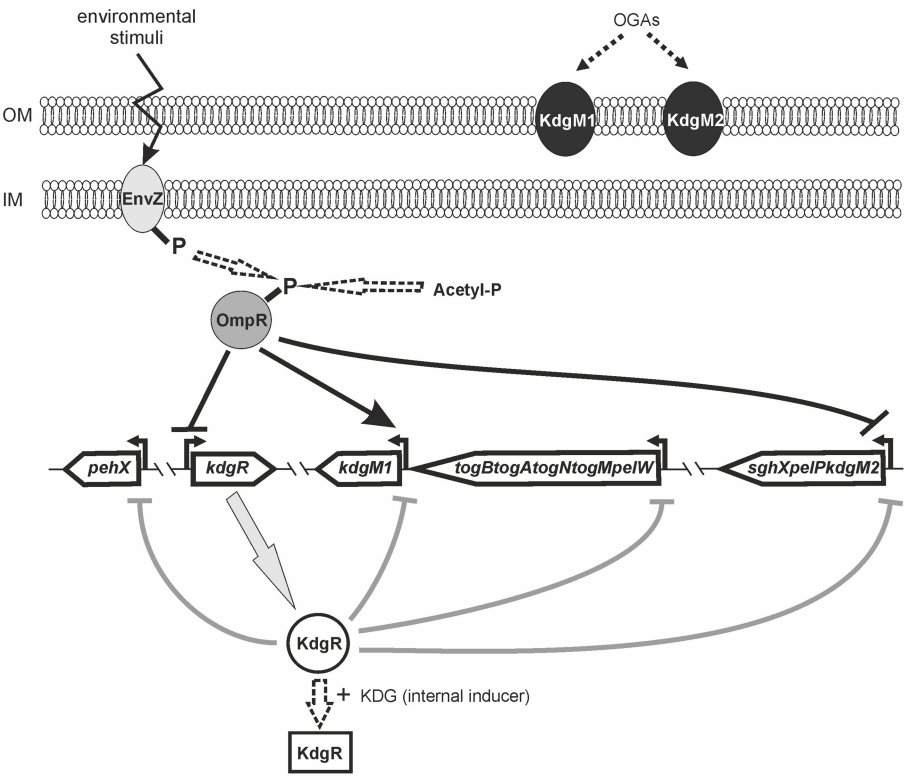

FIGURE 12 | Model of the OmpR and KdgR regulatory network controlling gene expression involved in the uptake and depolymerization of oligogalacturonides in *Y. enterocolitica*. Oligogalacturonides (OGAs) transported from the environment into *Y. enterocolitica* cells via the KdgM porins can be used as a carbon source. OGA uptake and catabolism are controlled at the transcriptional level by repressor KdgR. Binding of the OGA degradation product 2-keto-3-deoxygluconate (KDG) by KdgR leads to its inactivation, resulting in derepression of genes/operons of the KdgR regulon. The transcriptional regulation of *kdgM1*, *pehX*, *pelW-togMNAB*, and *kdgM2-pelP-sghX* expression is mediated by KdgR and OmpR regulators. OmpR activated (phosphorylated) by an unidentified environmental signal directly and/or indirectly regulates expression of the genes/operons of KdgR regulon. OmpR-P binding to the promoter regions of *kdgM1* and *kdgM2-pelP-sghX* leads to their activation and repression, respectively. Binding of OmpR-P to the *kdgR* regulatory region inhibits its transcription causing indirect upregulation of *kdgM1* and *kdgM2-pelP-sghX.* The expression of *pehX* and *pelW-togMNAB* is indirectly upregulated by OmpR-P. The different regulatory effects at the transcriptional level are indicated by an arrow for positive regulation or by a line with a bar for negative regulation. The possible roles of EnvZ and acetyl-phosphate in OmpR phosphorylation as well as KDG in the inactivation of KdgR are denoted by dotted arrows.

2007). Interestingly, no elements with sequence homology to the consensus OmpR-binding site were detected in the regulatory regions of the *D. dadantii kdgM* or *kdgN* genes (Condemine and Ghazi, 2007).

If OmpR negatively regulates *kdgR*, other genes of the *Y. enterocolitica* KdgR regulon should be indirectly influenced by OmpR. Using reporter gene fusion assays we found that the *pehX* gene, encoding the polygalacturonase PehX and the *pelW-togMNAB* operon, encoding the cytoplasmic exopolygalacturonate lyase PelW and oligogalacturonide transport system TogMNAB are positively regulated by OmpR. Both transcriptional units are members of the KdgR regulon. It is worth nothing that while we identified KdgR binding motifs in the respective promoter regions, we were unable to detect appropriate OmpR-binding sites. Thus, the regulatory effect of OmpR on *pehX* and *pelW-togMNAB* may be linked to its influence on *kdgR* expression. Together, our findings show that the effects of OmpR on some members of the KdgR regulon are likely to be direct, by transcriptional control of particular genes, and/or indirect, by modulation of the expression of other regulatory factor genes, including *kdgR* (see model, **Figure 12**.)

In the course of this study we verified the hypothesis that an OM channel formed by *Y. enterocolitica* KdgM2 might represent a route via which low molecular weight hydrophilic β-lactam antibiotics can enter the cell. However, neither upregulation of KdgM2 in the absence of the general porins OmpC and OmpF in the *ompR* mutant nor the upregulation of both KdgM1 and KdgM2 in the *kdgR* mutant, affected the antibiotic sensitivity of these strains, which argues against a role for these oligogalacturonide-specific porins in the penetration of the studied drugs. Interestingly, specific porins like maltoporin LamB and phosphate transport porin PhoE have been shown to contribute to resistance to certain β-lactam antibiotics in *Klebsiella pneumoniae* (Kaczmarek et al., 2006; Garcia-Sureda et al., 2011).

Interestingly, overexpression of KdgM2 in *Y. enterocolitica* was found to (i) increase outer membrane permeability, as revealed by the accumulation of hydrophobic dye NPN, and (ii) make cells more susceptible to the trimethoprim. These results corroborate the recent finding in *Salmonella* that an increase in KdgM proteins in a mutant strain lacking elongation factor P leads to increased OM permeability (Zou et al., 2011). However, contrary to the *Salmonella* data we were unable to demonstrate increased susceptibility to the gentamicin, probably due to the presence of a functional AcrAB efflux system in *Y. enterocolitica* (Raczkowska et al., 2015). Interestingly, the increased permeability of *Y. enterocolitica* membranes caused by overexpressed KdgM2 was only observed at 37°C and not at the normal growth temperature of 26°C. Notably, this raised temperature is also known to inhibit the synthesis of the O-polysacharide chain of lipopolysaccharide (LPS) in yersiniae cells (Bengoechea et al., 2004; Skurnik et al., 2007). Thus, the biological relevance of KdgM2 may be associated with growth of *Y. enterocolitica* at 37°C, i.e., the body temperature of a mammalian host. The tight negative regulation of *kdgM2* expression by OmpR would therefore be necessary to decrease OM permeability and prevent the influx of deleterious antimicrobial factors produced by the host.

Our data raised questions concerning the redundancy of KdgM porins in *Y. enterocolitica* and the adaptive role of OmpR associated with the modulation of their levels. The differential regulation of KdgM1 and KdgM2 by OmpR might reflect the varied function of these proteins in bacteria growing in different environmental niches, as has been proposed for OmpC and OmpF in *E. coli* (Nikaido, 2003). *Y. enterocolitica* exhibits a dual lifestyle, existing as both a non-pathogenic saprophyte and a pathogen residing inside the host body. These two environments differ greatly in the nature of the carbon and energy sources available and in the presence and concentration of harmful compounds. In the saprophytic lifestyle, *Y. enterocolitica* may utilize both KdgM porins to acquire OGAs from the surrounding environment. In the host body, upregulation of more specific channels may be necessary for the uptake of OGAs present in the intestinal environment (Cummings et al., 1979; Cummings and Englyst, 1987; Gibson et al., 1990). OGAs could be derived from pectin degradation mediated by the pectinolytic activity of symbiotic microbiota sharing the same ecological niche. On the other hand, the inhibition of KdgM2 production by OmpR might decrease OM permeability and limit the diffusion of harmful compounds into cells growing within a mammalian host. Thus, production of the appropriate KdgM porin for a particular local environment, mediated by the regulator OmpR, might contribute to the fitness of *Y. enterocolitica*.

AUTHOR CONTRIBUTIONS

MN designed and performed experiments, analyzed data and helped to write the paper. AR performed some experiments and analyzed data. KJ, ES, KS, and DS helped to perform some experiments. KB designed the experiments, analyzed data, wrote the paper and provided financial support.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fcimb.2017.00366/full#supplementary-material

Figure S1 | Amino acid sequence alignment of selected KdgM family members from *Yersiniae*, *D. dadantii*, and *P. carotovorum*. **(A)** The aligned amino acid sequences are KdgM1 and KdgM2 from *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (Y.ent.; Taxonomy ID: 994476), *Y. pseudotuberculosis* IP 32953 (Y.pse.; Taxonomy ID: 273123), *Y. pestis* KIM10+ (Y.pes.; Taxonomy ID: 187410), KdgN and KdgM from *D. dadantii* 3937 (D.dad.; Taxonomy ID: 198628), and KdgN, KdgM, KdgM3, and KdgM4 from *P. carotovorum* subsp. *carotovorum* PC1 (P.car.; Taxonomy ID: 561230). Identical residues are marked by asterisks, highly similar residues are denoted by colons and slightly similar residues are indicated by periods. The program T-Coffee (version 8.93) was used to draw the alignment and the sequence accession numbers are shown. **(B)** Percentage identity between

KdgM1/KdgM2 from *Y. enterocolitica* subsp. *palearctica* 105.5R(r) and the other KdgM proteins determined using Protein BLAST.

Figure S2 | Alignment of the KdgR amino acid sequence of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (Y.ent.O:9; Acc.no. ADZ42452) with those of *D. dadantii* 3937 (D.dad.;Acc. no. ADM98638) and *P. carotovorum* subsp. *carotovorum* PC1 (P.car.; Acc. no. ACT12926). Identical residues are marked by asterisks, highly similar residues are denoted by colons and slightly similar residues are indicated by periods. The HTH motif is boxed in gray. The program T-Coffee (version 8.93) was used to draw the alignment.

Table S1 | Strains and plasmids used in this study.

Table S2 | Oligonucleotide primers used in this study.

Additional File 1 | Bioinformatic analysis of *Y. enterocolitica* proteins of the KdgM family.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

A

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Y.ent.(ADZ42456)KdgM1 MKFKLLTLAVA-----SVI--SFSSVATTIDYRHEMKDTSKSDHKDRLLI
Y.ent.(ADZ44282)KdgM2 MKFKLLSLAVA-----SLI--STSTMAVTIDYRHETQDQSGNNHKDRLLM
Y.pse.(CAH21601)KdgM1 MKLKLITLAVAAAA-VSVI--SFSSVATTIDYRHEMKDTSKSDHKDRLLI
Y.pse.(CAH23073)KdgM2 MNIKLLSLAVA-----SLI--STSAVAVTIDYRHEMQDQSGNTHKDRLLM
Y.pes.(AAM85447)KdgM1 MKLKLITLAVAAAA-VSVI--SFSSVATTIDYRHEMKDTSKSDHKDRLLI
Y.pes.(AAM87378)KdgM2 MNIKLLSLAVA-----SLI--STSAVAVTIDYRHEMQDQSGNTHKDRLLM
D.dad.(ADM98428)KdgN  MKFKILTMMVA-----SVV--SMSSMAVTFDYRHEMKDTQNANHQDRLLI
D.dad.(ADM98622)KdgM  MKIKLLTLAVA-----SLV--SVNALAVSIDYRHEMQDTAQAGHKDRLLI
P.car.(ACT13042)KdgN  MKAKILTMMVT-----SLI--SLSSMAVTIDYRHEMKDTAKNDHKDRLLM
P.car.(ACT12944)KdgM  MKFKLLALAVT-----SLI--SVNAMAVTIDYRHEMQDTVKNDHKDRLSM
P.car.(ACT13207)KdgM3 MKIYTRNLLIVFAGFAPFLTQAETDGKTTFQYEHNWK-TEDRRHADSIKL
P.car.(ACT13208)KdgM4 MKKRYLVAGILFAQ-IYAI--SASASDTKLSYEHSWG-TMNRYHGDQIGM
                      *:       :        :  : .   ..:.*.*.        * * : :

Y.ent.(ADZ42456)KdgM1 SNRFANGFGLSLEGKWGQHSSD-TTPNKPFNEQVSNGTEVVASYVYQFNK
Y.ent.(ADZ44282)KdgM2 SHRFANGFGLSLEGKWKSAS----DKDKPFHEGVSNGTEVVASYVYKIDK
Y.pse.(CAH21601)KdgM1 SNRFANGFGLSLEGKWGQHSSD-TTPNKPFNEQVSNGTEMVASYVYKFNN
Y.pse.(CAH23073)KdgM2 SHRFANGFGLSLEGKWKGAQ----DKDKPFNETVSNGTEVVASYVYKINN
Y.pes.(AAM85447)KdgM1 SNRFANGFGLSLEGKWGQHSSD-TTPNKPFNEQVSNGTEMVASYVYKFNN
Y.pes.(AAM87378)KdgM2 SHRFANGFGLSLEGKWKGAQ----DKDKPFNETVSNGTEVVASYVYKINN
D.dad.(ADM98428)KdgN  SHRFENGFGLSSEVKWKQSSND-NTPNKPYNEQVSNGTEVTASYLYKFDK
D.dad.(ADM98622)KdgM  SHRFANGFGLSSEVKWAQSSAD-KTPNKPFNEQVSNGTEVVASYVYKFNS
P.car.(ACT13042)KdgN  SHRFDNGFGLSMEAKWGQSDSD-KTPNKPFNETVSNGTEVTASYLYKFDK
P.car.(ACT12944)KdgM  SHRFANGFGLSAEAKWKQSGSD-NTPNKPFNEPVSNGTEVVASYVYNFNK
P.car.(ACT13207)KdgM3 IHKKTNGW--SYEVKFSTSAGGNSNYDVAYDDMQGGSGGMVIGKDFKLSK
P.car.(ACT13208)KdgM4 RHFMDNGLYVGVELNFYNKNK-----DLTIDDVVSNSYAFYTGYAYSLTP
                       :   **   . * ::          : . .:  ...  .  .  :.:

Y.ent.(ADZ42456)KdgM1 TFQLEPGFSL-----------------ESSSDSNNYRPYLRGKVSFTDDF
Y.ent.(ADZ44282)KdgM2 TFSIEPGFSL-----------------DSSSTANSYRPYLRGKANITDDL
Y.pse.(CAH21601)KdgM1 TFQIEPGFSL-----------------DSTSDSNNYRPYIRGKVAFTDDF
Y.pse.(CAH23073)KdgM2 TFSIEPGFSL-----------------DSNSTSNNYRPYLRGKAVIVDDL
Y.pes.(AAM85447)KdgM1 TFQIEPGFSL-----------------DSTSDSNNYRPYIRGKVAFTDDF
Y.pes.(AAM87378)KdgM2 TFSIEPGFSL-----------------DSNSTSNNYRPYLRGKAVIVDDL
D.dad.(ADM98428)KdgN  MFNVEAGFNL-----------------VTDSSTNSYRPYIRGGVNFTDSL
D.dad.(ADM98622)KdgM  VFSIEPGFSL-----------------ESGSSNNNYRPYLRGRANVTDDL
P.car.(ACT13042)KdgN  TFGLEAGLNM-----------------VSNSSSNNYRPYIKGTANITDSL
P.car.(ACT12944)KdgM  TFSLEPGFSL-----------------DSSSDSNNYRPYLRGKINITDDL
P.car.(ACT13207)KdgM3 AATLTPSFEF-----------------SIGNASMMYQPGLKYNYRINSDW
P.car.(ACT13208)KdgM4 ELTLTPNLEARFYSGGTSGEGTVGDIGASQSSGARYTPGLKLTWAVTDKT
                        : ..:.                     .    * * ::    . ..

Y.ent.(ADZ42456)KdgM1 STSLRYRPYYKRNQPA---Q---TKGTEK----------------------
Y.ent.(ADZ44282)KdgM2 SVSLRYRPYYKRTSANINTK---KDTSEN----------------------
Y.pse.(CAH21601)KdgM1 STSLRYRPYYKRNQPA---Q---TKSTEK----------------------
Y.pse.(CAH23073)KdgM2 SVSLRYRPYYKRTSANINTP---KDTSEN----------------------
Y.pes.(AAM85447)KdgM1 STSLRYRPYYKRNQPA---Q---TKSTEK----------------------
Y.pes.(AAM87378)KdgM2 SVSLRYRPYYKRTSANINTP---KDTSEN----------------------
D.dad.(ADM98428)KdgN  YYTLRYRPFYKRYSGSINTTPPAEATNMK----------------------
D.dad.(ADM98622)KdgM  SVALRYRPYFKRNSGNIG-K---DNTMDK----------------------
P.car.(ACT13042)KdgN  YYGLRYRASYLRVSGDIGKSSVTNPTDIT----------------------
P.car.(ACT12944)KdgM  STSLRYRPYYLRKNANTTKTGPANYTKET----------------------
P.car.(ACT13207)KdgM3 STYGRYRYEYKKPTRSSRYS-TISTSDKYGHAGESYLSKSDTGRHRLDAG
P.car.(ACT13208)KdgM4 DLHAQYRYDLRKITRSKRTS---TDDDTH----------------------
                         :**    :

Y.ent.(ADZ42456)KdgM1 -------GHEFTMLFAYNFLKNYSAEYELNYKKSE--D--EILANKEKEE
Y.ent.(ADZ44282)KdgM2 -------GYNLTSVLSYKINKDFQLDYELDYKQANKAG--VILSDNENYD
Y.pse.(CAH21601)KdgM1 -------GHEFTMLFAYNFLKDYSAEYELNYKKSE--D--EILANKEKYE
Y.pse.(CAH23073)KdgM2 -------GYNLTSVISYKIAKDYQLDYEIDYKQANKAG--VVLADKENYD
Y.pes.(AAM85447)KdgM1 -------GHEFTMLFAYNFLKDYSAEYELNYKKSE--D--EILANKEKYE
Y.pes.(AAM87378)KdgM2 -------GYNLTSVISYKIAKDYQLDYEIDYKQANKAG--VVLADKENYD
D.dad.(ADM98428)KdgN  -------GYTITSVLGYKFLDNFTVEYELEYNKNTKAGNFGYIYDNDNDN
D.dad.(ADM98622)KdgM  -------GYTLTGNIDYTFLKDYTIGYELEYKKGTSGK--TILSDNDDYD
P.car.(ACT13042)KdgN  -------GYTITSTLGYKLPANFVVEYEFEYNKNNKAGAPWYLADSENYE
P.car.(ACT12944)KdgM  -------GYNLTAVVSYKFLKDYQIDYELDYKKANKAGPSGFQADKENWK
P.car.(ACT13207)KdgM3 VTYSGFDNINLTYVFNYYIGDNTTKSYKYSKGEFTERE--YAVYDNGKTD
P.car.(ACT13208)KdgM4 -------RHRYEAGVAYKGFGNFTLAYTAYYYHA---D--YVLQNNKKHD
                             . *    :    *     .          :. . .

Y.ent.(ADZ42456)KdgM1 WSHDLKVAYKWDKNWKPYVAIGNVAG-SKTTDERQTRYRVGVQYSF
Y.ent.(ADZ44282)KdgM2 WTHDFKLTYKWDKNWSPYMAIGNVSG-SKNTDERQTRYRVGIKYSF
Y.pse.(CAH21601)KdgM1 WAHDVKIAYKWDKNWKPYMAIGNVPG-SKVTDERQTRYRVGIQYSF
Y.pse.(CAH23073)KdgM2 WSHDLKLTYKWDKNWSPYMALGNVSG-SKNTDERQTRYRVGVKYSF
Y.pes.(AAM85447)KdgM1 WAHDVKIAYKWDKNWKPYMAIGNVPG-SKVTDERQTRYRVGIQYSF
Y.pes.(AAM87378)KdgM2 WSHDLKLTYKWDKNWSPYMALGNVSG-SKNTDERQTRYRVGVKYSF
D.dad.(ADM98428)KdgN  LTHDVKLAYKIDKNWTPYIQVANVEGPSKTTDERQTRYRVGVQYNF
D.dad.(ADM98622)KdgM  ITHNVKLSYKWDKNWKPYVEVGNVSG-SETTDERQTRYRVGVQYSF
P.car.(ACT13042)KdgN  MSHDIKLAYKVDKNWTPYAQIGNVKG-STVTDERQTRYRVGVQYSF
P.car.(ACT12944)KdgM  IEHDVKLAYKLDKNWKPYMAIGNVVG-DKNTDERQTRYRVGVQYSF
P.car.(ACT13207)KdgM3 YEHQFKVQYKLNKQLTPYIEYDDISQ-SSTSSSRQGKIKVGFNYVF
P.car.(ACT13208)KdgM4 YQQDFDVSYTINDNWTAHVGVEDVAS-GRDVKSREGKGKVGFTYTF
                        ::.: *. :.: ..:    ::   .   ..*: : :**. * *
```

B

	KdgM1 *Y.ent.*	KdgM2 *Y.ent.*
% identity to KdgM1 *Y.ent.*		62
% identity to KdgM2 *Y.ent.*	62	
% identity to KdgM1 *Y.pse.*	90	61
% identity to KdgM2 *Y.pse.*	64	88
% identity to KdgM1 *Y.pes.*	90	61
% identity to KdgM2 *Y.pes.*	64	88
% identity to KdgN *D.dad.*	57	57
% identity to KdgM *D.dad.*	65	65
% identity to KdgN *P.car.*	58	59
% identity to KdgM *P.car.*	67	71
% identity to KdgM3 *P.car.*	23	25
% identity to KdgM4 *P.car.*	23	22

Figure S1. Amino acid sequence alignment of selected KdgM family members from *Yersiniae*, *D. dadantii*, and *P. carotovorum*. **(A)** The aligned amino acid sequences are KdgM1 and KdgM2 from *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (Y.ent.; Taxonomy ID: 994476), *Y. pseudotuberculosis* IP 32953 (Y.pse.; Taxonomy ID: 273123), *Y. pestis* KIM10+ (Y.pes.; Taxonomy ID: 187410), KdgN and KdgM from *D. dadantii* 3937 (D.dad.; Taxonomy ID: 198628), and KdgN, KdgM, KdgM3, and KdgM4 from *P. carotovorum* subsp. *carotovorum* PC1 (P.car.; Taxonomy ID: 561230). Identical residues are marked by asterisks, highly similar residues are denoted by colons and slightly similar residues are indicated by periods. The program T-Coffee (version 8.93) was used to draw the alignment and the sequence accession numbers are shown. **(B)** Percentage identity between KdgM1/KdgM2 from *Y. enterocolitica* subsp. *palearctica* 105.5R(r) and the other KdgM proteins determined using Protein BLAST.

```
Y.ent.(ADZ42452) MAIADLDKQPDSVSSVLKVFGILQALGEEREIGITELSQRVMMPKSTVYR
D.dad.(ADM98638) MAIADLDKQPDSVSSVLKVFGILQALGEEREIGITELSQRVMMSKSTVYR
P.car.(ACT12926) MAIADLDKQPDSVSSVLKVFGILQALGEEREIGITELSQRVMMSKSTVYR
                 ******************************************.******
                                                      HTH motif
Y.ent.(ADZ42452) FLQTMKSLGYVAQEGESEKYSLTLKLFELGAKALQNVDLIRSADIQMREL
D.dad.(ADM98638) FLQTMKSLGYVAQEGESEKYSLTLKLFELGAKALQNVDLIRSADIQMREL
P.car.(ACT12926) FLQTMKSLGYVAQEGESEKYSLTLKLFELGAKALQNVDLIRSADIQMREL
                 ************************************************

Y.ent.(ADZ42452) SNETRETIHLGALDEDSIVYIHKIDSMYNLRMHSRIGRRNPLHSTAIGKV
D.dad.(ADM98638) SALTRETIHLGALDEDSIVYIHKIDSMYNLRMYSRIGRRNPLHSTAIGKV
P.car.(ACT12926) SALTRETIHLGALDEDGIVYIHKIDSMYNLRMYSRIGRRNPLHSTAIGKV
                 *  *************.***************:*****************

Y.ent.(ADZ42452) LLAWRDRGEVEEILSHIEFTRSTKHTLDSAAALLPVLDQVREQGFGEDVE
D.dad.(ADM98638) LLAWRDREEVKEILSQVEFKRSTVHTIGSTEELLPQLDLVRQQGYGEDNE
P.car.(ACT12926) LLAWRDRGEVAEVLSTVEFTRSTPHTLCTAEDLLNQLDVVRGQGYGEDNE
                 ******* ** *:** :**.*** **: ::  **  ** ** **:*** *

Y.ent.(ADZ42452) EQEEGLRCIAVPVFDRFGVVIAGLSISYPTLRFSEDSKSDIVKSLHRAAR
D.dad.(ADM98638) EQEEGLRCIAVPVFDRFGVVIAGLSISFPTIRFSEDNKHEYVAMLHTAAR
P.car.(ACT12926) EQEEGLRCIAVPVFDRFGVVIAGLSISFPTIRFSEENKHEYVAMLHTAAR
                 ****************************:**:****:.* : *  ** ***

Y.ent.(ADZ42452) NISEQMGYRDYPF
D.dad.(ADM98638) NISDQMGYHDYPF
P.car.(ACT12926) NISEQMGYHDYPF
                 ***:****:****
```

Figure S2. Alignment of the KdgR amino acid sequence of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (Y.ent.O:9; Acc.no. ADZ42452) with those of *D. dadantii* 3937 (D.dad.; Acc. no. ADM98638) and *P. carotovorum* subsp. *carotovorum* PC1 (P.car.; Acc. no. ACT12926). Identical residues are marked by asterisks, highly similar residues are denoted by colons and slightly similar residues are indicated by periods. The HTH motif is boxed in gray. The program T-Coffee (version 8.93) was used to draw the alignment.

Table S1. Strains and plasmids used in this study.

Strains and plasmids	Description	Reference or source
***Y. enterocolitica* O:9**		
Ye9	wild-type, clinical isolate of serotype O:9, carrying virulence plasmid pYV	Clinical isolate, laboratory collection
Ye9N	Ye9 derivative, spontaneous Nal^R mutant	Brzostek et al., 2007
Ye9NK1	Ye9N derivative carrying a *kdgM1::lacZYA'* fusion, Nal^R, Cm^R	This work
ES1	Ye9N derivative, Δ*kdgR*::Gm defective in KdgR production, Nal^R, Gm^R	This work
ES1K1	ES1 derivative carrying a *kdgM1::lacZYA'* fusion, Nal^R, Gm^R, Cm^R	This work
MN1	Ye9N derivative, Δ*kdgM2*::Gm defective in KdgM2 production, Nal^R, Gm^R	This work
AR4	Ye9N derivative Δ*ompR*::Km defective in OmpR production, Nal^R, Km^R	Brzostek et al., 2003
AR4K1	AR4 derivative carrying a *kdgM1::lacZYA'* fusion, Nal^R, Km^R, Cm^R	This work
AR11	AR4 derivative Δ*kdgR*::Gm defective in KdgR production, Nal^R, Km^R, Gm^R	This work
AR11K1	AR11 derivative carrying a *kdgM1::lacZYA'* fusion, Nal^R, Km^R, Gm^R, Cm^R	This work
AR10	AR4 derivative, Δ*kdgM2*::Gm defective in KdgM2 production, Nal^R, Km^R, Gm^R	This work
E. coli		
S17-1 λpir		Simon et al., 1983
TOP10 F'		Invitrogen
DH5α		Sambrook et al., 1989
BL21 (DE3)		Life Technologies
TG1		Sambrook et al., 1989
W	ATTC 9637	ATTC Collection
Other strains		
Pectobacterium carotovorum subsp. *carotovorum* PCM 2056	wild-type strain	PCM Collection*
Rhizobium etli CE3	spontaneous Sm^R derivative of wild-type strain CFN42	Noel et al., 1984
PLASMIDS		
pDrive	cloning vector, Ap^R, Km^R	Qiagen
pFUSE	suicide vector, derivative of pEP185.2 with promoterless *lacZYA* genes, Cm^R	Baumler et al., 1996
pFkdgM1	pFUSE with XbaI/SmaI fragment (549-bp) of *kdgM1*, Cm^R	This work
pBluescript II SK(+)	*ori* M13, *ori* pBR322, general cloning vector, Ap^R	Stratagene
pBluescript/ *kdgM2'-'rfp*	pBluescript II SK (+) carrying 264 bp upstream of the *kdgM2* start codon and 10 codons of *kdgM2* fused in frame with *rfp*, Ap^R	This work
pBBR1MCS-5	broad-host-range cloning vector, *ori* pBBR1, Mob^+, *oriT* RK2, Gm^R	Kovach et al., 1995

pBKRFP	pBBR1MCS-5 derivative carrying *kdgM2'-'rfp* cloned into BamHI and EcoRI sites	This work
p34E-Tp	source of trimethoprim cassette, Ap^R,Tp^R	Deshazer and Woods, 1996
pBKRFP-Tp	pBKRFP derivative carrying Tp^R cassette from p34E-Tp inserted into the SalI site	This work
pDS132	*ori* R6K (narrow host range, replication only in *E. coli* λpir), *oriT* RK2, *sacB*, Cm^R	Philippe et al., 2004
pDSkdgR	pDS132 derivative carrying 2196-bp cassette for *kdgR* mutagenesis constructed by overlap extension PCR cloned between XbaI sites of the vector, Gm^R	This work
pDSkdgM2	pDS132 derivative carrying 2186-bp cassette for *kdgM2* mutagenesis constructed by overlap extension PCR cloned between XbaI sites of the vector, Gm^R	This work
pCM132Gm	pCM132 derivative, *ori* pMB1, *ori* RK2, *oriT* RK2, promoterless *lacZ* gene, Gm^R	DBG collection**
pCM132Gm-*kdgR::lacZ*	pCM132Gm derivative carrying 479 bp upstream of *kdgR* start codon and 95 bp of ORF *kdgR* cloned upstream of promoterless *lacZ* gene between EcoRI and KpnI sites	This work
pCM132Gm-*pehX::lacZ*	pCM132Gm derivative carrying 628 bp upstream of *pehX* start codon and 77 bp of ORF *pehX* cloned upstream of promoterless *lacZ* gene between EcoRI and KpnI sites	This work
pCM132Gm-*pelW-togMNAB::lacZ*	pCM132Gm derivative carrying 594 bp upstream of *pelW* start codon and 108 bp of ORF *pelW* cloned upstream of promoterless *lacZ* gene between EcoRI and KpnI sites	This work
pETOmpR	pET28a carrying the entire *ompR* coding sequence (725-bp fragment), Km^R	Nieckarz et al., 2016
pHR4	pHSG575 with 740-bp fragment of *ompR* (ORF with rbs), Cm^R	Brzostek et al., 2003
pBR3	pBBR1MCS-3 with XhoI/PstI fragment containing entire coding sequence of *ompR* (ORF with rbs), Tet^R	Brzostek et al., 2007
pHSG575	low copy number cloning vector, Cm^R	Takeshita et al., 1987
pkdgR-Cm	pHSG575 with BamHI/HindIII fragment containing entire coding sequence of *kdgR* (ORF with rbs), Cm^R	This work
pBBR1MCS-3	broad-host-range cloning vector, *ori* pBBR1, Mob^+, *oriT* RK2, Tet^R	Kovach et al., 1995
pkdgR-Tet	pBBR1MCS-3 with KpnI/SacI fragment containing entire coding sequence of *kdgR* (ORF with rbs), Tet^R	This work
pBAD18Km	Arabinose-regulated expression plasmid, Km^R	Guzman et al., 1995
pBAD-kdgM2	pBAD18Km with SacI/SphI fragment containing entire coding sequence of *kdgM2* (ORF with rbs), Km^R, *kdgM2* under the control of the inducible pBAD promoter	This work
pRK2013	helper plasmid used to mobilize vectors in triparental mating, Km^R	Ditta et al., 1980

*PCM Collection, Polish Collection of Microorganisms - Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (ECCO)

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Cm^R chloramphenicol resistance, Gm^R gentamicin resistance, Km^R kanamycin resistance, Nal^R nalidixic acid resistance, Sm^R streptomycin resistance, Tet^R tetracycline resistance, ::Km, insertion of kanamycin resistance cassette; ::Gm, insertion of gentamicin resistance cassette

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Table S2. Oligonucleotide primers used in this study.

Purpose and Target	Name of primer	Primer sequence (5'→ 3')*	Restriction enzyme	Reference
RT-PCR *kdgM2-pelP*	RTkdgMpelP1	AAAGCGCCAGTGACAAAGAT		This study
	RTkdgMpelP2	ATCAACAAACCGGTGTCCTC		This study
RT-PCR *pelP-sghX*	RTpelPsghX1	CCTGGCCGATCAGTATGTTT		This study
	RTpelPsghX2	TTTCCAGACCTTCAGCCAAC		This study
EMSA *kdgM1*	kdgM1x	**TGTCTAGA**ATCCTTCTGTTGCCGGTTTA	XbaI	This study
	kdgM1s	**TGCCCGGG**TTACAGATGCCACTGCCAGA	SmaI	This study
EMSA *kdgM2*	k2M1	**ACTCTAGA**TGTGTTAGCTTCCCTCACTGG	XbaI	This study
	k2M500	**ACCCCGGG**AGTTCCGAGATCCATGACTA	SmaI	This study
EMSA *kdgR*	kdgReA	CACGACACCTCATGGAAGG		This study
	kdgReB	ACCTATTTCCCGTTCTTCACCC		This study
Negative control for EMSA 16S rDNA	16SR1	ATTCCGATTAACGCTTGCAC		Nieckarz et al., 2016
	16SR304	GTGGGGTAATGGCTCACCTA		
Construction of *kdgR* mutants	KdgR1	**GCTCTAGA**ATGCTCGCATGTGGCTAATC	XbaI	This study
	KdgR2	CATCCGTTTCCACGGGTTACCCTACTCA		This study
	KdgR3	TGAGTAGGGTAACCCGTGGAAACGGATG		This study
	KdgR4	AAGATCCCTCAGAACGATCTCGGCTTGA		This study
	KdgR5	TCAAGCCGAGATCGTTCTGAGGGATCTT		This study
	KdgR6	**CGTCTAGA**GTAACATCCGCTCAGTGAA	XbaI	This study
	KdgR0	TGGGTGCCGTTGACGGATTG		This study
	KdgR7	GGCCAGGGTCAGACTTTCTC		This study
Construction of *kdgM2* mutants	KdgM1	**GCTCTAGA**CCACTTCGCCATACTTTGGT	XbaI	This study
	KdgM2	CTTCATCCGTTTCCACGCATAGCTGGCAACCACT		This study
	KdgM3	AGTGGTTGCCAGCTATGCGTGGAAACGGATGAAG		This study
	KdgM4	GCTGCCGGAAACATTACCGATCTCGGCTTGAACG		This study
	KdgM5	CGTTCAAGCCGAGATCGGTAATGTTTCCGGCAGC		This study
	KdgM6	**CGTCTAGA**GGGCATTCCAAAAACCACGAAT	XbaI	This study
	KdgM0	AATCACTGGGCTTTAGTCGAA		This study
	KdgM7	ATCGGTTTGCCATATTCACC		This study
Confirmation the correctness	LL1	ATTTAATTCGAAGGCGATCC		This study
	RR4	CGAAGGTGAGCCAGTGTGAC		This study

of *kdgM2'-'rfp* fusion.				
Construction of *kdgR::lacZ* transcriptional fusion	KdgREcoRI	**TAGAATTC**ATGATGGTTCGTTGATGGTG	EcoRI	This study
	KdgRKpnI	**TAGGTACC**TTCCCGTTCTTCACCCAAT	KpnI	This study
Construction of *pelW-togMNAB::lacZ* transcriptional fusion	PelWEcoRI	**TAGAATTC**GCTGTCATGGGTGTAACTCGT	EcoRI	This study
	PelWKpnI	**TAGGTACC**GCTGTGGCTTACACACTGGA	KpnI	This study
Construction of *pehX::lacZ* transcriptional fusion	PehXEcoRI	**TAGAATTC**AGAAAAAGAGTGGCGTCTCG	EcoRI	This study
	PehXKpnI	**TAGGTACC**ATCGGAGTACCCACCATCAG	KpnI	This study
Confirmation the correctness of fusions constructed in pCM132Gm	pCM132GmSPR1	CTGCAAGGCGATTAAGTTGG		This study
	pCM132GmSPR2	CATAAACTGCCAGGCATCAA		This study
Construction of *kdgM1::lacZ* transcriptional fusion	KdgM1X	**TGTCTAGA**ATCCTTCTGTTGCCGGTTTA	XbaI	This study
	KdgM1S	**TGCCCGGG**TTACAGATGCCACTGCCAGA	SmaI	This study
Confirmation the correctness of fusion constructed using pFUSE	LPkdgM2683	CACAGATGCTTTCCATTGGT		This study
	lacZH991	CATCGCAGGCTTCTGCTTC		This study
Control of contamination with genomic DNA	Y1	AATACCGCATAACGTCTTCG		Wannet et al., 2001
	Y2	CTTCTTCTGCGAGTAACGTC		
Construction of pkdgR-Cm	KdgRorfBamHI	**TAGGATCC**TGAAACACAGAAAACTGAGTAGGG	BamHI	This study
	KdgRorfHindIII	**TAAAAGCTT**CCCTAGTGGAAAGATCCCTCA	HindIII	This study
Construction of pkdgR-Tet	KdgRorfKpnI	**TAAGGTACC**TGAAACACAGAAAACTGAGTAGGG	KpnI	This study
	KdgRorfSacI	**TAGAGCTC**CCCTAGTGGAAAGATCCCTCA	SacI	This study
Construction of pBAD-KdgM2	ARAkdgM2SacI	**TGAGCTC**GTTATTTATTAAAATAAGGTAACTGTAATG	SacI	This study
	ARAkdgM2SphI	**TATAAGCATGC**TAGCGAGGTGTGACCCCTAA	SphI	This study
RT-qPCR	16SrRNA-F	CACACTGGAACTGAGACA		This study
	16SrRNA-R	TGCTTCTTCTGCGAGTAA		This study
	kdgM2-F	CTTATCAGCACCAGCACAAT		This study
	kdgM2-R	ACCGTTACTCACACCTTCA		This study
	kdgR-F	TCGTGGTGAAGTAGAAGAGAT		This study
	kdgR-R	CAGGCACAGCGATACAAC		This study
	pelP-F	AAGTCAATATAGACACCACCAACAG		This study

	pelP-R	TATGGCATCAACATCGGCAT A	This study

The table shows the sequences of primers used for cloning, the construction of translational and transcriptional fusions, and to generate fragments for protein-DNA binding assays (EMSAs).
*- 5' extensions added to introduce cleavage sites for the indicated restriction enzymes **are *shown in bold.***

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Wannet, W.J., Reessink, M., Brunings, H.A, and Maas, H.M. (2001). Detection of pathogenic *Yersinia enterocolitica* by a rapid and sensitive duplex PCR assay. *J. Clin. Microbiol*. 39, 4483–4486. doi: 10.1128/JCM.39.12.4483-4486.2001

Additional File 1. Bioinformatic analysis of *Y. enterocolitica* proteins of the KdgM family.

Bioinformatic analysis of *Y. enterocolitica* proteins of the KdgM family

Bioinformatic analysis of the *Y. enterocolitica* subsp. *palearctica* 105.5R(r) (3/O:9 bio-serotype) genome (NCBI Reference Sequence: NC_015224.1) revealed the presence of two KdgM homologs: one encoded by the *kdgM1* gene (Acc. no. ADZ42456; nucleotide position 2,265,584 bp to 2,264,886 bp), with an ORF of 699 bp (232 aa protein including signal peptide), and the other by the *kdgM2* gene (Acc. no. ADZ44282; nucleotide position 4,365,460 bp to 4,364,756 bp), with an ORF of 705 bp (234 aa protein including signal peptide). The mature KdgM family porins of *Y. enterocolitica* subsp. *palearctica* 105.5R(r) are proteins of similar size: KdgM1 – 24,817 Da, KdgM2 – 24,724 Da.

Amino acid sequence alignment of KdgM1 and KdgM2 revealed 62% identity (**Supplementary Figure S1**). Both KdgM proteins of *Y. enterocolitica* exhibit 65% identity to KdgM and 57% identity to KdgN, the two oligogalacturonide-specific porins of *D. dadantii* (**Supplementary Figure S1**). Genes encoding KdgM1 and KdgM2 were identified in the genomes of another subspecies of *Y. enterocolitica*, subsp. *enterocolitica* 8081 (99% amino acid identity to KdgM1 and KdgM2 of subsp. *palearctica* 105.5R(r)) and also in other pathogenic *Yersiniae*, i.e. *Y. pseudotuberculosis* IP 32953 (KdgM1, 63% amino acid identity to KdgM and 54% amino acid identity to KdgN; KdgM2, 65% amino acid identity to KdgM and 53% amino acid identity to KdgN) and *Y. pestis* (CO92 and KIM10+) (KdgM1, 63% amino acid identity to KdgM and 54% amino acid identity to KdgN; KdgM2, 65% amino acid identity to KdgM and 53% amino acid identity to KdgN). Four KdgM homologs (KdgM, KdgN, KdgM3, KdgM4) present in the genome of *Pectobacterium carotovorum*, (formerly *Erwinia carotovora*; Rodionov et al., 2004) exhibit different levels of identity to *Y. enterocolitica* KdgM1 and KdgM2, with KdgM3 and KdgM4 being the least similar (**Supplementary Figure S1**).

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Źródła finansowania

- Grant NCN OPUS UMO-2011/01/B/NZ6/01845: Badania proteomiczne i genetyczne w celu określenia roli szlaku sygnałowego EnvZ/OmpR *Yersinia enterocolitica* w regulacji właściwości wirulentnych oraz adaptacji do warunków stresowych

  Kierownik projektu – prof. dr hab. Katarzyna Brzostek

- Grant uniwersytecki DSM 107433: Rola szlaku sygnałowego EnvZ/OmpR w regulacji syntezy adhezyny YadA *Yersinia enterocolitica* O:9

  Kierownik projektu – mgr Marta Nieckarz

- Grant uniwersytecki DSM 110129: Analiza funkcji dwuskładnikowego szlaku transdukcji sygnału EnvZ/OmpR w regulacji ekspresji receptora błony zewnętrznej HemR *Yersinia enterocolitica* O:9

  Kierownik projektu – mgr Marta Nieckarz

- Grant uniwersytecki DSM 86-0115000-29: Analiza oddziaływań białka OmpR z obszarem regulatorowym genów białek błony zewnętrznej KdgM1 i KdgM2 oraz ich represora KdgR u *Yersinia enterocolitica* O:9

  Kierownik projektu – mgr Marta Nieckarz

Oświadczenia współautorów

Publikacja oryginalna 1

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek

Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR

Environmental Microbiology, 2016, 18: 997–1021.
doi:10.1111/1462-2920.13165

Impact factor: 5.395
Punktacja MNiSW: 40

Warszawa, 24.11.2017

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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na:
1. zaplanowaniu prowadzonych prac eksperymentalnych (wraz z prof. Katarzyną Brzostek i dr hab. Adrianną Raczkowską),
2. analizach proteomicznych i genetycznych, w tym: (i) optymalizacji protokołu izolacji frakcji białek osłon komórkowych wzbogaconych o białka błony zewnętrznej (wraz z dr. Januszem Dębskim); (ii) przygotowaniu preparatów peptydowych do analiz MS; (iii) bioinformatycznej analizie danych otrzymanych w doświadczeniach proteomicznych; (iv) analizach SDS-PAGE i Western blot dla białka YadA i HemR; (v) konstrukcji fuzji reporterowej *hemR-lacZYA'* i plazmidu pompR do badania komplementacji mutacji *ompR*; (vi) badaniu poziomu transkrypcji *hemR*; (vii) nadprodukcji i oczyszczeniu białka OmpR-His_6; (viii) badaniu wiązania białka OmpR z sekwencjami regulatorowymi *yadA* i *hemR* (testy EMSA),
3. dyskusji otrzymanych wyników (wraz z prof. K. Brzostek i z dr hab. A. Raczkowską),
4. przygotowaniu manuskryptu (wraz z prof. K. Brzostek), przygotowaniu rycin załączonych do pracy (z wyłączeniem figur 4, 5A, 6B i 7A) oraz dodatkowych materiałów dostępnych *on-line*.

Badania te realizowałam w ramach pracy doktorskiej wykonywanej w Zakładzie Mikrobiologii Stosowanej Wydziału Biologii UW.

Marta Nieckarz

podpis

Warszawa, 24.11.2017

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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na współautorstwie koncepcji badań (wraz z Martą Nieckarz i prof. Katarzyną Brzostek), przeprowadzeniu analiz bioinformatycznych w celu wskazania potencjalnych miejsc wiązania białka OmpR, analizach poziomu fluorescencji szczepów niosących fuzję YadA'–'GFP metodą cytometrii przepływowej (wraz z dr Ombeline Rossier), interpretacji wyników badań (wraz z M. Nieckarz i prof. K. Brzostek), graficznym opracowaniu rycin 4, 5A, 6B i 7A.

.....

podpis

Warszawa, 24.11.2017

Dr Janusz Dębski
Środowiskowe Laboratorium Spektrometrii Mas
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Polska Akademia Nauk

OŚWIADCZENIE

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Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na optymalizacji warunków uzyskania preparatów peptydowych do różnicowej analizy proteomicznej (wraz z Martą Nieckarz).

.....

podpis

Warszawa, 24.11.2017

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Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na przeprowadzeniu analizy głównych składowych (ang. Principle Component Analysis, PCA).

M. Kistowski

.....

podpis

Warszawa, 24.11.2017

Prof. dr hab. Michał Dadlez
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Instytut Genetyki i Biotechnologii
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Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na konsultacjach naukowych dotyczących różnicowych analiz proteomicznych.

.....
podpis

Munich, 24.11.2017

Prof. dr Jürgen Heesemann
Max von Pettenkofer Institute for Hygiene and Medical Microbiology
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CO-AUTHOR STATEMENT

I hereby certify that in the publication:

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

my contribution comprised consultations on issues concerned YadA and HemR expression and providing rabbit antisera directed against HemR and YadA.

07/12/2017

signature

Prof. Dr. Dr. J. Heesemann
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Dr. Ombeline Rossier
Associate Professor

CO-AUTHOR STATEMENT

I hereby certify that, in the publication:

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

my contribution comprised constructing a strain carrying a GFP translational fusion with YadA, monitoring bacterial fluorescence by flow cytometry (together with Dr. hab. Adrianna Raczkowska) and revising the manuscript critically for important intellectual content on YadA and HemR expression.

Dr. Ombeline Rossier

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Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Janusz Dębski, Michał Kistowski, Michał Dadlez, Jürgen Heesemann, Ombeline Rossier, Katarzyna Brzostek (2016) **Impact of OmpR on the membrane proteome of *Yersinia enterocolitica* in different environments: repression of major adhesin YadA and heme receptor HemR.** *Environmental Microbiology*, 18: 997–1021. doi:10.1111/1462-2920.13165

mój udział polegał na opracowaniu koncepcji badań, nadzorowaniu i koordynowaniu prowadzonych prac eksperymentalnych, analizie i interpretacji wyników (wraz z Martą Nieckarz i dr hab. Adrianną Raczkowską), przygotowaniu manuskryptu (wraz z M. Nieckarz), pozyskaniu finansowania badań (grant NCN nr 2011/01/B/NZ6/01845).

.....
podpis

Publikacja oryginalna 2

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek

The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*

Frontiers in Cellular and Infection Microbiology, 2017, 7: 366.
doi:10.3389/fcimb.2017.00366

Impact factor: 4.3
Punktacja MNiSW: 40

Warszawa, 24.11.2017

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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na:
1. zaplanowaniu prowadzonych prac eksperymentalnych (wraz z prof. Katarzyną Brzostek i dr hab. Adrianną Raczkowską),
2. analizach genetycznych i fizjologicznych, w tym: (i) bioinformatycznej analizie genów/białek związanych z transportem i katabolizmem produktów degradacji pektyn; (ii) izolacji i analizie SDS-PAGE preparatów białek osłon komórkowych; (iii) analizie aktywności fuzji translacyjnej *kdgM2'-'rfp*; (iv) mutagenezie genu *kdgM2*; (v) konstrukcji fuzji reporterowych *kdgR::lacZ*, *pehX::lacZ* i *pelW-togMNAB::lacZ* oraz analizie ich aktywności; (vi) konstrukcji szczepów niosących fuzję *kdgM1-lacZYA'* (wraz z Dorotą Stosio) oraz analizie aktywności *kdgM1-lacZYA'* w podłożu minimalnym; (vii) konstrukcji plazmidów pkdgR-Cm i pkdgR-Tet do komplementacji mutancji *kdgR*; (viii) konstrukcji plazmidu pBAD-KdgM2 do nadekspresji białka KdgM2; (ix) analizach RT-PCR i RT-qPCR; (x) analizach zdolności szczepów bakteryjnych do produkcji pektynaz, wrażliwości szczepów *Y. enterocolitica* na gentamycynę i trimetoprim oraz detergenty; (xi) badaniu poziomu reaktywnych form tlenu oraz maceracji tkanek roślinnych (wraz z dr hab. A. Raczkowską); (xii) nadprodukcji i oczyszczeniu białka OmpR-His_6; (xiii) badaniu wiązania białka OmpR z sekwencjami regulatorowymi *kdgM1*, *kdgM2* i *kdgR* (wraz z D. Stosio),
3. dyskusji otrzymanych wyników (wraz z dr hab. A. Raczkowską i prof. K. Brzostek),
4. przygotowaniu manuskryptu (wraz z prof. K. Brzostek), przygotowaniu rycin (z wyłączeniem rycin 11B i 12) oraz dodatkowych materiałów załączonych do pracy dostępnych *on-line*.

Badania te realizowałam w ramach pracy doktorskiej wykonywanej w Zakładzie Mikrobiologii Stosowanej Wydziału Biologii UW.

Marta Nieckarz
.....
podpis

Warszawa, 24.11.2017

Dr hab. Adrianna Raczkowska
Zakład Mikrobiologii Stosowanej, Instytut Mikrobiologii
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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na współautorstwie koncepcji badań (wraz z Martą Nieckarz i prof. Katarzyną Brzostek), przeprowadzeniu analiz bioinformatycznych w celu wskazania potencjalnych miejsc wiązania białka OmpR, analizie poziomu reaktywnych form tlenu i zdolności szczepów *Y. enterocolitica* do maceracji tkanek roślinnych (wraz z M. Nieckarz), a także badaniu przepuszczalności osłon komórkowych, dyskusji otrzymanych wyników (wraz z M. Nieckarz i prof. Katarzyną Brzostek), graficznym opracowaniu rycin 11B i 12.

.....

podpis

Warszawa, 24.11.2017

Karolina Jaworska
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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na: analizie wrażliwości szczepów *Y. enterocolitica* na antybiotyki β-laktamowe i tetracyklinę.

Badania te wykonałam pod opieką Marty Nieckarz w ramach pracowni magisterskiej realizowanej w Zakładzie Mikrobiologii Stosowanej Wydziału Biologii UW.

Karolina Jaworska
.....
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Warszawa, 24.11.2017

Ewa Stefańska
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Wydział Biologii, Uniwersytet Warszawski

OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na mutagenezie genu *kdgR* w szczepach *Y. enterocolitica* oraz utworzeniu plazmidów pBKRFP i pBKRFP-Tp niosących fuzję *kdgM2'-'rfp* i wprowadzeniu ich do szczepów *Y. enterocolitica.*

Badania te wykonałam z pomocą i pod opieką dr Karoliny Skorek w ramach pracy magisterskiej realizowanej w Zakładzie Mikrobiologii Stosowanej Wydziału Biologii UW.

Ewa Stefańska
.....
podpis

Warszawa, 24.11.2017

Dr Karolina Skorek
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Wydział Biologii, Uniwersytet Warszawski

OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na analizie bioinformatycznej sekwencji nukleotydowych w celu otrzymania delecji genu *kdgR* w szczepach *Y. enterocolitica* oraz utworzeniu plazmidów pBKRFP i pBKRFP-Tp niosących fuzję *kdgM2'-'rfp* (wraz z Ewą Stefańską).

Badania te wykonałam podczas studiów doktoranckich na Wydziale Biologii UW.

K Skorek

.....

podpis

Warszawa, 24.11.2017

Dorota Stosio
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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na konstrukcji szczepów niosących fuzję *kdgM1-lacZYA'* (wraz z Martą Nieckarz) oraz badaniu poziomu transkrypcji *kdgM1* w podłożu LB, a także badaniu wiązania białka OmpR z sekwencjami promotorowymi *kdgM1*, *kdgM2* oraz *kdgR* (wraz z M. Nieckarz).

Badania te wykonałam z pomocą i pod opieką Marty Nieckarz w ramach pracy magisterskiej realizowanej w Zakładzie Mikrobiologii Stosowanej Wydziału Biologii UW.

Dorota Stosio
podpis

Warszawa, 24.11.2017

Dr hab. Katarzyna Brzostek, prof. UW
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OŚWIADCZENIE

Oświadczam, że w pracy:

Marta Nieckarz, Adrianna Raczkowska, Karolina Jaworska, Ewa Stefańska, Karolina Skorek, Dorota Stosio, Katarzyna Brzostek (2017) **The role of OmpR in the expression of genes of the KdgR regulon involved in the uptake and depolymerization of oligogalacturonides in *Yersinia enterocolitica*.** *Frontiers in Cellular and Infection Microbiology*, Aug 15;7:366. doi:10.3389/fcimb.2017.00366

mój udział polegał na opracowaniu ogólnej koncepcji badań, nadzorowaniu i koordynowaniu badań, dyskusji otrzymanych wyników (wraz z Martą Nieckarz i dr hab. Adrianną Raczkowską), przygotowaniu manuskryptu (wraz z M. Nieckarz), pozyskaniu finansowania badań (grant NCN nr 2011/01/B/NZ6/01845).

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podpis